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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

COMMENTARY

Methotrexate and Central Nervous System Toxicity [1,2]

Herbert T. Abelson [3,*]

With the advent of more aggressive treatment protocols involving methotrexate (MTX), central nervous system (CNS) toxicity has become a more pressing issue. The combination of intrathecal MTX plus irradiation is effective therapy for CNS prophylaxis in acute lymphoblastic leukemia (1) and childhood lymphoblastic lymphoma.[4] However, MTX and cranial irradiation combined are primarily responsible for the clinical and pathologic changes associated with the various forms of neurotoxicity.

These CNS complications range from abnormal computerized tomographic scans in asymptomatic patients (2) to severe encephalopathy (3,4). The encephalopathy may be either acute or delayed, and occurs most commonly in patients treated with cranial irradiation in addition to intrathecal and/or parenteral MTX. In its most severe form, the encephalopathy may become progressive and include seizures, ataxia, stupor, paraplegia, dementia, and death. Pathologic changes have included multi-focal necrosis of white matter, fibrinoid changes in adjacent blood vessels, and periventricular calcifications (5–8).

The mechanisms underlying the clinical and pathologic changes associated with cranial irradiation and MTX are unknown. When MTX is preceded by or accompanies cranial irradiation, the clinical changes may be more profound including severe and at times progressive encephalopathy as previously noted. Irradiation may directly damage endothelial cells altering MTX permeability (9). Irradiation alone may produce mineralizing microangiopathic changes in the cortical grey matter (10) which are not produced by intrathecal or iv MTX alone (11). An important consideration is whether MTX is directly cytotoxic to the brain, ie, results in cell death within the brain. MTX only exerts its cytotoxic effects on cells in cycle. Resting cells (G_0) are completely resistant to the effects of MTX (12). Neurons are G_0 cells and do not replicate; therefore, MTX should not be cytotoxic towards them, although it may derange their function (see below). However, there are tissues within the brain such as glial (13) and endothelial (14) cells which do replicate. The microglial or scavenger cells are responsible for macrophage-like functions within the CNS. Death of these cells would result in inefficient removal of metabolic waste products and cellular debris from the adjacent neural elements. The fact that glial and endothelial cell replication might be inhibited by MTX may be of particular importance when cranial irradiation and MTX are used concomitantly. Cranial irradiation may cause direct damage to the blood-brain barrier by affecting endothelial cells or their junctions (10,11). Therefore, endothelial cell replication may be inhibited by two different mechanisms and may allow increased concentrations of MTX to enter the CNS (15).

At the biochemical level, there are at least two important pathways in CNS metabolism which might be deranged by MTX. MTX is a potent inhibitor of the enzyme dihydrofolate reductase (DHFR), its putative cytotoxic target (16,17). DHFR is responsible for maintaining the intracellular pool of reduced folate co-factors (tetrahydrofolates) which are required for the de novo synthesis of purine nucleotides and thymidylate. This enzyme has been identified and partially characterized in both normal mammalian brain (18–20) and human brain tumors (21). Inhibition of DHFR by MTX may, in addition to inhibiting de novo nucleic acid biosynthesis and thereby replicative functions, result in an intracellular buildup of oxidized folates, ie, dihydrofolate which might be toxic to the brain (18).

MTX may also exhibit inhibitory effects within a completely different biochemical setting (fig 1). Tetrahydrobiopterin is a required co-factor for hydrox-

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[3]Department of Pediatrics, Harvard Medical School; Division of Hematology-Oncology, Children's Hospital Medical Center; and the Sidney Farber Cancer Institute, Boston, Mass.

[4]Weinstein HJ, Vance ZB, Jaffe N, et al. Improved prognosis for patients with mediastinal lymphoblastic lymphoma. Manuscript in preparation.

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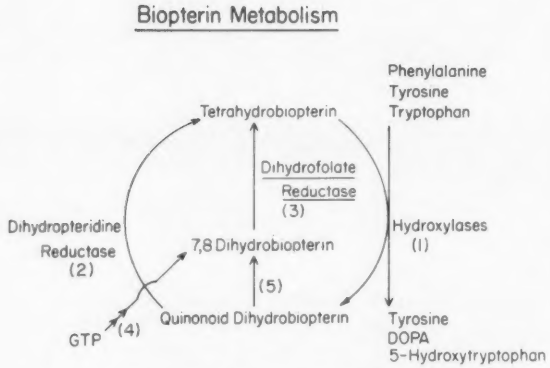

FIGURE 1.—Schematic interactions in tetrahydrobiopterin metabolism.

ylation reactions involving biogenic amines and serotonin (22,23). The conversions of phenylalanine to tyrosine, tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and tryptophan to 5-hydroxytryptophan (5-HT) all involve hydroxylase reactions with absolute requirements for tetrahydrobiopterin as co-factor (reaction 1). There are two pathways by which tetrahydrobiopterin may be formed. Tetrahydrobiopterin may be formed from the quinonoid form of dihydrobiopterin by the action of dihydropteridine reductase (reaction 2, regenerative pathway) (24). Dihydropteridine reductase is found within all regions of the brain (25) and is partially inhibited by MTX (Ki, 38 μM) (24). Tetrahydrobiopterin may also be formed in the brain (20,26,27) as in the liver (24) by reduction of 7,8-dihydrobiopterin to tetrahydrobiopterin (reaction 3). This reaction involves DHFR and is completely inhibited by MTX (20,28). 7,8-Dihydrobiopterin may be formed in the brain de novo from guanosine-5′-triphosphate (29,30) (reaction 4) and may be nonenzymatically produced from quinonoid dihydrobiopterin (28) (reaction 5). The amount of tetrahydrobiopterin in the brain may therefore be regulated by biochemical pathways which include DHFR and are susceptible to inhibition by MTX (26). Patients with childhood leukemia or psoriasis treated with MTX have been reported to have increased biopterin levels in their serum as detected by *Crithidia fasciculata* and increased dihydroxanthopterin in their urine (31,32). Similar changes are reported for serum and urine from patients with dihydropteridine reductase deficiency (31). Also, where it has been assayed in liver, the phenylalanine hydroxylase system is indirectly inhibited in vivo and in vitro by the effect of MTX on tetrahydrobiopterin regeneration via dihydropteridine reductase (33).

In adults receiving high-dose MTX, somnolence and confusion accompany drug administration in about 10% of patients.[5] Decreased availability of biogenic amines and serotonin might lead to these changes in mentation and, together with abnormalities resulting from inhibition of the replicative pathways involving DHFR, may combine to produce various degrees of encephalopathy. Seizures and neurologic deterioration in a patient with dihydropteridine reductase deficiency have been accompanied by low concentrations of biogenic amines in the CNS (34). Replacement therapy with L-DOPA and 5-HT in this patient resulted in increased brain levels of serotonin and dopamine. Other patients have been similarly treated (35,36), and 5-HT has also been used as replacement therapy in other neurologic disorders (37,38). The administration of choline has also been shown to stimulate tyrosine hydroxylase activity in rats (39) as well as providing a protective effect against MTX-induced chronic liver changes (40).

It may be possible to directly answer whether cerebrospinal fluid biogenic amine levels are influenced by MTX since a sensitive high-pressure liquid chromatographic assay has recently been made available.[6] Measuring biopterin levels in serum and cerebrospinal fluid could provide further evidence, although indirect, that tetrahydrobiopterin regeneration is impaired after MTX administration (20,24,28,31,32). Similarly, measuring biopterin levels in patients receiving triamterene (an inhibitor of dihydropteridine reductase) (41) would be of interest.

There are, therefore, a number of etiologic factors possibly responsible for the CNS toxicity associated with MTX: (*a*) direct effect of MTX on the replicative pathways involving DHFR resulting in cell death; (*b*) decreased availability of some neurotransmitters; and (*c*) potentiation of MTX effects by irradiation.

A general rethinking of how best to prevent neurologic side effects of antifols is needed. At present, management of these complications of therapy involves adjusting MTX and citrovorum factor schedules or dosages. If abnormalities in biopterin synthesis or metabolism can be documented, then serious consideration should also be given to replacement therapy with L-DOPA (34–36), 5-HT (34–38), or choline (39,40).

[5]Ensminger W, and Abelson HT. Unpublished observations.
[6]Wick M. Personal communication.

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COMMENTARY

Pulmonary Toxicity of Antineoplastic Drugs [1]

James K. V. Willson [2,3,*]

SUMMARY

Drug-induced pulmonary disease is an infrequent, but clinically significant, toxic manifestation of antineoplastic drug therapy. Pulmonary toxicity has been associated primarily with bleomycin therapy but also has been described with busulfan, cyclophosphamide, methotrexate, and the nitrosoureas. Cytotoxic drug-induced pulmonary disease is characterized by an insidious and nonspecific presentation. The common pathologic process of a fibrosing alveolitis follows a variety of toxic mechanisms associated with the chemotherapeutic agents. The importance of close clinical monitoring is emphasized by the necessity for early withdrawal of the drug in order to halt or reverse potentially lethal pulmonary disease. At the same time, the nonspecific nature of the lung disease and the multitude of potential insults to the lung in a cancer patient make an early aggressive evaluation mandatory. A lung biopsy is almost always indicated.

[Cancer Treat Rep 62:2003–2008, 1978]

There has been an increased awareness of the pulmonary toxicity associated with many cancer chemotherapeutic agents (1–3). At present, bleomycin is the only chemotherapeutic agent with a high incidence of related pulmonary toxicity (4). The recent availability and widespread use of bleomycin have heightened our awareness of the potential of antineoplastic drugs for causing a spectrum of pulmonary diseases. In addition, the increasingly aggressive diagnostic and supportive care which cancer patients receive has led to identification of previously undiagnosed pulmonary diseases, a number of which appear to be drug-related. The importance of early recognition of these cytotoxic drug-induced diseases is emphasized by the necessity for early withdrawal of the drug in order to halt or reverse potentially lethal progression of pulmonary damage (3). At the same time, it is often difficult to differentiate drug-induced toxicity from toxicity of other potential insults in this group of patients. These insults include metastatic or primary malignancy, an opportunistic infection, pulmonary edema, pulmonary emboli, and pulmonary reaction to therapeutic and diagnostic procedures. The nonspecific manifestations of all these potential insults mandate an early lung biopsy once a diffuse interstitial pulmonary disease develops in a patient receiving antineoplastic drug therapy (5). The characteristic clinical presentation, pathophysiology, and clinical course of the lung diseases associated with bleomycin, busulfan, cyclophosphamide, methotrexate, and the nitrosoureas will be reviewed.

BLEOMYCIN

Bleomycin, an antibiotic isolated from *Streptomyces verticillus*, has assumed a major role in the management of non-Hodgkin's lymphomas (6), Hodgkin's disease (7), testicular cancer (8), and carcinoma of the head and neck (9). The limiting toxic effect of bleomycin is the interstitial pneumonitis reported to occur in 5%–20% of patients receiving bleomycin (4,9–12). This wide range reflects different definitions of toxicity and, perhaps, varia-

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tions in incidence related to different schedules of administration. Bleomycin-induced pneumonitis has been documented by lung biopsy in 5% of patients receiving bleomycin (4). A death rate of 1% has been reported in most large series evaluating bleomycin therapy (4).

A number of parameters which increase the risk of bleomycin pulmonary toxicity have been identified. These include the total dose administered, age, and concomitant antineoplastic therapy. With total doses of bleomycin > 450 mg, the incidence and severity of pulmonary toxicity are proportional to the total dose of drug (4). A 10% death rate due to pulmonary insufficiency has been documented in patients receiving a total dose > 550 mg (4). There is no direct dose-toxicity function at a total dose < 450 mg; however, severe pulmonary toxicity has occasionally been documented with total doses of bleomycin < 100 mg (9–12). Age is a factor associated with an increased frequency of pulmonary toxicity. This has been documented by the observation of greater toxicity with bleomycin therapy in patients in their 60s and 70s (4,9). Concomitant radiotherapy appears to potentiate the pulmonary toxicity of bleomycin. A controlled study (13) reported severe pulmonary fibrosis occurring in five of 13 patients receiving bleomycin combined with concomitant radiation therapy of 2000 rads to the lung primary. A similar study (14) reported seven of 16 patients who developed severe pneumonitis with bleomycin doses between 120 and 240 mg and radiotherapy of 5000–6000 rads to the lung primary. In another series (15), five of 12 patients with a prior history of lung exposure to radiation therapy developed bleomycin pneumonitis. The increased use of bleomycin in combination with other chemotherapeutic agents has resulted in a substantial increase in the risk of developing bleomycin-induced pneumonitis. Severe pulmonary fibrosis developed in four of 16 patients with diffuse histiocytic lymphoma who were treated with 45–165 mg of bleomycin in combination with adriamycin, cyclophosphamide, vincristine, and prednisone (6). There is no clear association between the method of administration of bleomycin and its clinical toxicity (9). However, Samuels and his colleagues observed a lower incidence of interstitial pneumonitis than expected with a continuous infusion of high-dose bleomycin in the management of testicular cancer (8). In a mouse model for bleomycin-induced pneumonitis reported elsewhere in this issue (16), a continuous infusion of bleomycin resulted in less pulmonary toxicity than an intermittent injection of an equivalent dose of bleomycin. In this model, it is the peak tissue concentration of bleomycin which appears to determine pulmonary toxicity. This is a provocative study which should stimulate further clinical trials to define an optimal schedule for bleomycin therapy.

Histologic changes associated with bleomycin-induced pneumonitis include a nonspecific interstitial pneumonitis with fibrinous exudate, atypical proliferation of type II alveolar epithelial cells, and squamous metaplasia of the terminal bronchioles. Fibrosis is often extensive and irreversible. A distinctive feature of bleomycin-induced pneumonitis is the preferential distribution of pneumonitis in the lower lobes and subpleural regions (17).

Bleomycin-induced pneumonitis has been extensively studied in animal models (18–23) and appears to be a result of direct cytotoxic injury to lung epithelium followed by a fibrosing alveolitis (18). The cytotoxic mechanism of bleomycin has not been clearly elucidated, but bleomycin has been shown to fragment DNA. This fragmentation of DNA is felt to result from free-radical generation following binding of bleomycin to DNA (24). The enhancement of free-radical generation by radiotherapy and other antineoplastic drugs, such as adriamycin, may explain the increased toxicity resulting from combinations of such agents with bleomycin. The site of the initial cytotoxic insult is debated. A serial ultrastructural study in a mouse model of bleomycin-induced pneumonitis suggests capillary endothelial injury as the initial site of toxicity (18–20). Alternately, similar studies in an avian model (21) and in man (25) show an early epithelial cell injury. In addition to this cytotoxic damage, it has been proposed that bleomycin directly stimulates a fibroblastic proliferation. Ultrastructural studies have demonstrated the synthesis of immature collagen fibrils by fibroblasts in early bleomycin-induced pneumonitis (21). Biochemical analyses of lung have shown increased hydroxyproline (23) and collagen (22) formation in early bleomycin-induced lung disease. The lung lacks the inactivation enzymes specific for bleomycin (26), and this deficiency may explain the retention of bleomycin in the lung and the unique susceptibility of the lung to bleomycin toxicity.

The clinical presentation of bleomycin-induced pneumonitis is nonspecific; it is characterized by the occurrence of a nonproductive cough, dyspnea, and, at times, fever. The onset of clinical symptoms is insidious and occurs between 4 and 10 weeks after initiation of therapy. Tachypnea and diffuse rales are prominent features of the physical examination (4,9–13). Radiographic changes are characterized by bilateral basilar infiltrates which may progress to a diffuse interstitial pneumonitis. Pleural effusion is rare (27). Pulmonary function abnormalities include hypoxemia, a restrictive ventilatory defect, and a decreased diffusing capacity for carbon

monoxide (28). Clinical signs of pneumonitis are insensitive parameters for monitoring the pulmonary toxicity of bleomycin. These findings indicate that toxicity is well-established (9–13). The value of serial pulmonary function tests for predicting the pulmonary toxicity of bleomycin has not been established. However, one prospective study of patients receiving bleomycin found deterioration in pulmonary functions of asymptomatic patients with normal chest radiographs (28). This study suggests that clinically inapparent lung disease may be revealed by monitoring pulmonary function. Another useful study for early detection of bleomycin-induced pneumonitis is the gallium-67 lung scan, which is positive at the early stages of pneumonitis when the chest roentgenogram is normal (29). The specificity of the gallium-67 scan is limited, however, because it does not differentiate drug-induced pneumonitis from other inflammatory processes. The absence of specific parameters which herald the onset of bleomycin pulmonary injury makes it imperative that close attention be given to serial evaluation of the clinical examination, as well as to the pulmonary function tests and the chest roentgenogram in patients receiving bleomycin. Significant abnormalities should be evaluated by a lung biopsy.

The successful management of bleomycin-induced pneumonitis is dependent on early discontinuance of bleomycin. Steroid therapy has been reported to be effective in individual cases (10). The severe toxicity resulting from interstitial and alveolar fibrosis is irreversible.

BUSULFAN

Busulfan, an alkylating agent, is commonly used in the long-term management of chronic myelogenous leukemia (30). Following the initial case reports of Oliner et al (31), an interstitial pneumonitis, "busulfan lung," has been recognized as an infrequent, but serious and often fatal, complication of the long-term use of busulfan (31–36). A characteristic feature of busulfan lung is the long interval between the initiation of busulfan therapy and the onset of pneumonitis. The mean time to manifestation of pulmonary toxicity is 4 years, with a range of 8–10 years (36). A reliable estimate of the incidence of busulfan lung has not been established, but it is an infrequent toxic manifestation despite the ubiquitous use of busulfan. Twenty-four cases of busulfan-induced pneumonitis have been reported (34–36).

The insidious onset of a dry, nonproductive cough and dyspnea is the characteristic clinical presentation of busulfan-induced pneumonitis. The low-grade fever with associated constitutional symptoms may accompany the pulmonary symptoms. Tachypnea, diffuse rales, and cyanosis, in the severest cases, are noted on the physical examination. The chest roentgenogram often shows a diffuse intra-alveolar or interstitial process or a combination of both (31–37). The pulmonary functions of patients receiving long-term busulfan therapy have been monitored in a prospective study (38). This study demonstrated the sensitivity of the diffusion capacity, which was diminished in a significant number of patients without clinical symptoms. The development of clinical symptoms is insidious, with a long asymptomatic period and progression to a severe restrictive lung disease. Once clinical symptoms are manifested, pulmonary fibrosis is irreversible (35). The mean survival, following the onset of symptoms and diagnosis of pulmonary fibrosis, is 5 months (35).

Histologic changes in busulfan-induced pneumonitis include atypia of the alveolar and bronchiole epithelia with a background of interstitial and intra-alveolar edema and fibrosis. A predominantly central distribution of these parenchymal changes has been emphasized (32). The pathologic changes are believed to reflect the cytotoxic drug effect on the alveolar epithelium and capillary endothelium. An alveolitis results with subsequent interstitial and alveolar edema and eventual fibrosis. The alveolar epithelial atypia is manifested by hyperplasia of type II pneumonocytes (39). These type II pneumonocytes are exfoliated into the sputum of patients with busulfan lung, and, therefore, sputum cytologies are valuable in evaluating suspected busulfan-induced pneumonitis (36). This epithelial dysplasia is not restricted to pulmonary epithelium in prolonged busulfan therapy. Significant epithelial dysplasia following prolonged therapy with busulfan has been described in the cervix, pancreas, and gastrointestinal and urinary tracts (39). The possibility that this dysplasia is a premalignant change has been raised, and a number of carcinomas have been reported in patients receiving long-term busulfan therapy (40).

The clinical course of the respiratory deterioration is variable and may be stabilized with the early withdrawal of busulfan. Successful treatment with corticosteroids has been reported but has never definitely been shown to be beneficial (33,34).

CYCLOPHOSPHAMIDE

Cyclophosphamide-induced pneumonitis has been described in seven patients receiving continuous low-dose cyclophosphamide therapy. The duration of therapy in these case reports was between 1 and 36 months (41–47). The clinical symptoms described

in these patients included dyspnea, nonproductive cough, and fever. The onset of symptoms was insidious. Cyclophosphamide may produce a histopathologic picture similar to that described in the busulfan lung, and an identical mechanism of damage to alveolar epithelial cells is proposed to explain the cyclophosphamide-induced pneumonitis. A drug-induced alveolitis results with fibroblastic proliferation and eventual intra-alveolar fibrosis. Atypical type II pneumonocytes noted in other drug-induced pulmonary diseases are also a prominent feature of the histopathology described in cyclophosphamide-induced pneumonitis (41). Cyclophosphamide pneumonitis has been described in patients receiving continuous low-dose cyclophosphamide therapy and has not been associated with high-dose cyclophosphamide (48). Three of the seven reported patients died of respiratory failure while four recovered with minimal residual deficit after cyclophosphamide was discontinued.

METHOTREXATE

Methotrexate, a folate antagonist, is used extensively in the management of an increasing number of neoplastic, dermatologic, and immunologic disorders (49). The pulmonary toxicity associated with methotrexate is the most benign and, at the same time, the most controversial of those considered here. An extensive review of methotrexate-induced pneumonitis which includes the largest series of cases with methotrexate-associated pulmonary toxicity has recently appeared (50). This review found no relationship to dose or route of administration in methotrexate-induced pneumonitis. The incidence of pulmonary toxicity associated with methotrexate is not well-defined, but pneumonitis appears to be rare. The Yale series documents seven cases of methotrexate-induced pneumonitis in 93 patients treated with methotrexate at the Yale-New Haven Medical Center. The high incidence of pneumonitis attributed to methotrexate in the Yale series stands in contrast to the complete absence of pulmonary toxicity attributed to methotrexate in adjuvant breast chemotherapy trials (51) and in high-dose methotrexate studies (52).

The characteristics of the pneumonitis attributed to methotrexate therapy include a clinical presentation typical for an interstitial pneumonitis with fever and peripheral blood eosinophilia. The clinical course is generally self-limited, and recovery over a short period is the rule. Interestingly, persistent exposure to methotrexate does not alter the prognosis. Corticosteroids may shorten the recovery period in retrospective data but do not influence the overall prognosis (50,53).

Radiographic and pulmonary function abnormalities seen in methotrexate-induced pneumonitis are characteristic of a diffuse, interstitial pneumonitis (54). The impaired diffusion capacity is the most useful parameter to follow. Pathologic changes include an interstitial infiltrate of mononuclear cells. In contrast to the other cytotoxic drug-induced lung diseases, cellular atypia and fibrosis are rarely noted. Granulomatous changes have been described as part of the inflammatory process in three cases (50).

The etiology of the pneumonitis attributed to methotrexate therapy continues to be questioned. A direct cytotoxic mechanism has not been supported by pathologic studies (50). Animal models have never shown pulmonary toxicity resulting from methotrexate administration (55,56). Alternative explanations for the pneumonitis associated with methotrexate include the possible existence of an as yet undiagnosed insult to the lung, such as an opportunistic virus, or a unique sensitivity to methotrexate in man. A hypersensitivity reaction has also been suggested as a mechanism, and this notion is supported by the frequency of eosinophilia and the absence of a dose-toxicity relationship (50). On the other hand, the existence of a hypersensitivity reaction is challenged by the fact that methotrexate-associated pneumonitis did not recur with rechallenge by methotrexate in 13 patients (50) and that no pulmonary toxicity has been reported in the high-dose methotrexate studies (52). The impurity of past pharmaceutical preparations of methotrexate has led to the speculation that a contaminant has been responsible for methotrexate-associated pneumonitis. It has been emphasized previously that the physician must aggressively search for an alternative explanation in the setting of suspected drug-induced pneumonitis. This caution is especially true in a setting of suspected methotrexate-induced pneumonitis, the very existence of which remains in doubt.

THE NITROSOUREAS

The nitrosoureas are an important group of synthetic antineoplastic agents employed primarily as single agents in melanoma and gastrointestinal cancer as well as in combination drug therapy for lymphomas (57). In addition to the major toxic effect of cumulative bone marrow suppression, a severe pulmonary fibrosis has been described in 11 patients after prolonged therapy with BCNU (58–63). This toxic effect of BCNU therapy occurred following a total dose of 2–4 g given over a period of 6 months to 3 years. In all the reported cases, a diffuse, nonspecific, interstitial pulmonary fibrosis devel-

oped. Severe respiratory failure resulted, and six of the patients died of respiratory failure. In animal experiments, other nitrosoureas in addition to BCNU produced lethal pulmonary disease (57). The etiology of this toxicity has not been explained, but it is likely to be a response to the alkylating action of nitrosoureas on respiratory epithelium (60). The injury to respiratory epithelium results in an alveolitis and fibrosis. Pulmonary fibrosis is now recognized as a potential toxic effect of the nitrosoureas. This toxic effect may increase in frequency as these agents continue to be used in adjuvant therapy and as part of combination chemotherapy for responsive or curable malignancies such as Hodgkin's disease.

OTHER ANTINEOPLASTIC DRUGS

There have been isolated but well-documented reports of drug-induced interstitial pneumonitis following treatment with 6-mercaptopurine (64,65), azathioprine (66), melphalan (67), chlorambucil (68), mitomycin C (69), and procarbazine (70). Each case resulted in pulmonary fibrosis following a presumed cytotoxic insult to the lung parenchyma, except in the case of procarbazine where the toxic effect was clearly an allergic reaction.

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COMMENTARY

New Treatments for Malignant Melanoma [1]

James K. Luce [2,*]

DTIC and the nitrosoureas have become the primary drugs used in the chemotherapy for malignant melanoma. Most patients are treated with one or both of these agents before becoming subjects for investigative treatment.

Recent trials of new drugs or drug combinations in previously treated patients with melanoma have resulted in very low response rates. Many investigators have felt that previously treated melanoma patients are not suitable subjects for further chemotherapy trials. That this is an unwarranted pessimism is demonstrated by Bellet et al in this issue of *Cancer Treatment Reports*. Their 25% response rate with the single agent dibromodulcitol in patients who had previously received both DTIC and a nitrosourea is most gratifying and will lead to trials with this agent in previously untreated melanoma patients. Whether dibromodulcitol will prove to be consistently effective in the treatment of melanoma is, of course, not yet known.

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The important message carried in their report is that previously treated melanoma patients are excellent subjects for phase II and selected phase III drug trials. We are, after all, looking for drugs that are better than DTIC or the nitrosoureas. The ideal patient in whom to try a new regimen is one with a relatively resistant disease such as melanoma. If these usually refractory patients show even a low response rate, the regimen under trial is worthy of further testing.

Melanoma patients have the additional assets of being, on the average, younger and in better condition, and often have more easily measured pulmonary or subcutaneous metastases than patients with other kinds of cancer.

Dr. Bellet and his colleagues are to be congratulated for their productive efforts with previously heavily treated melanoma patients as subjects for phase II study. We can now feel justified in offering these patients the hope that accompanies a new trial of therapy instead of the depression that comes from being rejected because their disease is judged to be refractory to any drug treatment.

Improved Therapeutic Index of Bleomycin When Administered by Continuous Infusion in Mice [1]

Branimir Ivan Sikic,* Jerry M. Collins, Edward G. Mimnaugh, and Theodore E. Gram [2,3]

SUMMARY

The effects of three different dosage schedules on both therapeutic effect and pulmonary toxicity of bleomycin were studied in mice. Therapy was assessed by both survival and decreased tumor size in mice bearing Lewis lung carcinoma. Lung toxicity was estimated in nontumored mice as increases in lung collagen content by measuring lung hydroxyproline concentrations. In the first set of experiments, bleomycin injections twice daily (low-dose, high-frequency) produced a significant (34%) increase in lifespan over controls, whereas the same total dose given twice weekly did not result in increased survival. Both schedules produced pulmonary toxicity. Continuous sc infusion of bleomycin via an osmotic minipump was compared to these two schedules of intermittent injection. Identical total doses of drug were administered in all three schedules. Continuous infusion for 7 days produced marked inhibition of tumor growth (T/C = 16%), which was significantly better than twice weekly or ten-times weekly injection of the same total dose. Furthermore, at a total dose of 40 mg/kg of bleomycin, continuous infusion did not result in measurable pulmonary toxicity, whereas both schedules of bolus injection produced significant increases in lung collagen content. Thus, continuous infusion of bleomycin improved its therapeutic effect against Lewis lung carcinoma and also reduced its pulmonary toxicity.

[Cancer Treat Rep 62:2011–2017, 1978]

Bleomycin has been conventionally administered as an iv bolus or occasionally as an im injection (1). However, several clinical studies have been performed in which the drug is given by continuous iv or intra-arterial infusion for as long as 7 days (2–7). In these studies, continuous infusion seemed to improve the therapeutic efficacy of the drug when compared to historical controls using bleomycin injection, without increasing pulmonary toxicity. However, no randomized, prospective clinical trials comparing infusion to injection of bleomycin have been reported. Furthermore, assessment of pulmonary toxicity by gross clinical signs and symptoms is semi-quantitative at best, and overlooks "subclinical" toxicity which may well be present.

The rationale for bleomycin infusion is based on work by Drewinko et al (8) on human lymphoma cells in culture, in which increasing the duration of exposure to bleomycin from 4 to 35 hours decreased the survival fraction from 10% to 0.06%. In addition, the plasma half-life (elimination phase) of bleomycin in man is quite short, being < 2 hours after either injection or infusion, with rapid renal clearance and virtually no protein binding in plasma (8–10). Thus, a prolonged infusion might be expected to exert a

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greater antitumor effect than bolus injection, if, as has been suggested, the duration of treatment proved more important than peak levels of drug.

We have recently demonstrated in the mouse that lung collagen content measured by hydroxyproline levels is increased in chronic bleomycin lung toxicity, and that this increase correlated with dose-dependent changes in four quantifiable histopathologic parameters (11). We have now applied this model, along with treatment of Lewis lung carcinoma, to examine the effects of various schedules of bleomycin administration on its therapeutic index.

METHODS

Animals

Male BDF_1 mice (Taconic Farms, Inc, Germantown, NY), weighing 20–25 g at the start of the experiments, were used throughout and were maintained on Purina rodent chow and water ad libitum. Animals that were not implanted with tumors were used in all experiments assessing bleomycin pulmonary toxicity. Animals bearing implanted Lewis lung carcinoma cannot be used to assess lung toxicity because of their short lifespan and pulmonary metastases. Therefore, tumor-bearing animals were only used to measure therapeutic efficacy of bleomycin and are described separately.

Tumor Therapy

Male BDF_1 mice were implanted sc with Lewis lung carcinoma (Arthur D. Little, Inc, Cambridge, Mass). The tumor was prepared by mincing and forming a suspension containing 10^7 tumor cells/ml. Cell viability was determined by trypan blue exclusion, and 0.1 ml, or 10^6 cells, was injected sc into the left flank of each animal. Bleomycin treatment began on Day 4 after implantation, regardless of subsequent scheduling, and the effects of treatment were assessed by recording the time of survival from implantation and by measuring the length and width of the primary tumor at various time points.

Drug Dosage and Schedules

Bleomycin as the mixture of glycopeptides (lot U11A2) was obtained from Nippon Kayaku, Inc, Japan, through the courtesy of Dr. John Douros of the National Cancer Institute. The drug was dissolved in saline and administered sc in the right flank.

A total of four experiments were performed as follows:

Experiment 1A.—This experiment consisted of treatment of Lewis lung carcinoma, comparing intermittent higher-dose injection (10 mg/kg twice weekly for 4 weeks) with more frequent, lower-dose injection (2 mg/kg ten times weekly for 4 weeks).

Experiment 1B.—This experiment measured bleomycin pulmonary toxicity, with the drug schedules and dosages used in experiment 1A, in nontumored animals. There were 30–32 mice in each group and control animals received 0.9% NaCl twice weekly. Mice were killed 10 weeks after the initiation of treatment for determination of lung hydroxyproline content.

Experiment 2A.—In this experiment the effects of continuous sc infusion of bleomycin on growth of Lewis lung carcinoma were compared to the two intermittent injection schedules used in experiments 1A and B. Continuous sc infusion at a constant rate over a 7-day period was achieved with an Alzet osmotic minipump (Alza Corp, Palo Alto, Calif). These pumps, which measured 6.5 × 25 mm, were implanted under the skin through an incision just posterior to the right scapula, and were left in place for the duration of the experiment. Treatment began on Day 4 for all three schedules, so that the two-injection groups were treated on Days 4 and 7 after tumor implantation and the ten-injection groups were treated on Days 4–8. Dose-response curves were derived by treating animals in each of the three schedules with total doses of 10, 20, 40, and 80 mg/kg of bleomycin over a 1-week treatment period. Thus, there were a total of 12 treatment groups of ten animals each. In addition to an untreated control group, ten animals were implanted with minipumps containing 0.9% NaCl to control possible effects of a subcutaneous foreign body on tumor growth.

Experiment 2B.—In this experiment the animals without tumors were treated for 1 week with the three schedules of bleomycin used in experiment 2A, and were killed 10 weeks later for measurement of lung collagen content as an index of toxicity. Total doses of 10, 20, and 40 mg/kg of bleomycin were compared in all three schedules. Doses > 40 mg/kg which were given over 1 week by intermittent injection produced lethal toxicity in animals before the 10-week endpoint for measurement of lung collagen; 40 mg/kg over 1 week was the highest dose used in this experiment. An additional group of ten mice received 40 mg/kg of bleomycin over 2 weeks by infusion to examine the effect of prolonging an infusion while keeping the total dose constant.

Bleomycin Stability

Before implantation of minipumps containing bleomycin, the stability of solutions of the drug

incubated at 37°C was determined. There is a correlation between DNA strand-scission caused by bleomycin and its antimicrobial efficacy (12). We therefore incubated solutions of bleomycin, 0.1 and 1.0 mg/ml in 0.9% NaCl in capped vials and withdrew aliquots for measurement of antibiotic activity at 0, 1, 4, and 7 days, using the method of Pittillo et al (13).

Measurement of Lung Toxicity

We have previously shown that increases in lung collagen content, as measured by hydroxyproline levels, occur in chronic bleomycin pulmonary toxicity in mice, and are related to the dose of bleomycin and to changes in various histopathologic parameters (11). In both experiments assessing pulmonary toxicity, collagen content of the whole right lung was the primary parameter utilized. In addition, in experiment 1B, four morphometric histopathologic parameters were measured in the left lung of each animal, when lung collagen content did not reveal differences between the two treatment groups. The morphometric indices used were percent lung consolidation, total pulmonary cell count, number of intra-alveolar cells, and alveolar wall thickness. These methods have been described in detail (11).

RESULTS

Frequent injection of relatively small doses of bleomycin (2 mg/kg ten times weekly) produced a significant increase in lifespan of mice bearing Lewis lung carcinoma compared to controls (table 1). The same total dose of bleomycin twice weekly did not significantly prolong lifespan. However, both regimens were pneumotoxic, as demonstrated by increases in lung hydroxyproline levels and by measurements of percent consolidation of lung parenchyma (table 2). Neither these two parameters of lung toxicity nor three other morphometric parameters demonstrated an advantage for either schedule of treatment. However, there was an increase in lethal toxicity in the frequent, low-dose group, with eight of 32 deaths occurring over 10 weeks compared to one of 32 occurring in the 10-mg/kg twice weekly group ($P < 0.05$, chi-square 2×2 contingency table with Yates' correction). Thus, although therapeutic activity was slightly improved by more frequent injection, lung toxicity was approximately equivalent in the two groups.

These two schedules of injection were then compared to continuous infusion of bleomycin over 7 days using the implantable Alzet minipump. It was determined in preliminary experiments that bleomycin in 0.9% NaCl was stable for at least 7 days at 37°C. Figure 1 presents measurements of sizes of Lewis lung carcinoma on Day 15 after implantation for all three schedules over a range of total doses. Continuous infusion of bleomycin was clearly superior to the two schedules of intermittent injection in inhibiting tumor growth. The therapeutic advantage of low-dose, more frequent treatment over twice weekly treatment was confirmed in this experiment ($P < 0.05$, Student's t test, at 20, 40, and 80 mg/kg total doses). A control group of tumor-bearing mice implanted with minipumps containing 0.9% NaCl did not differ from untreated controls.

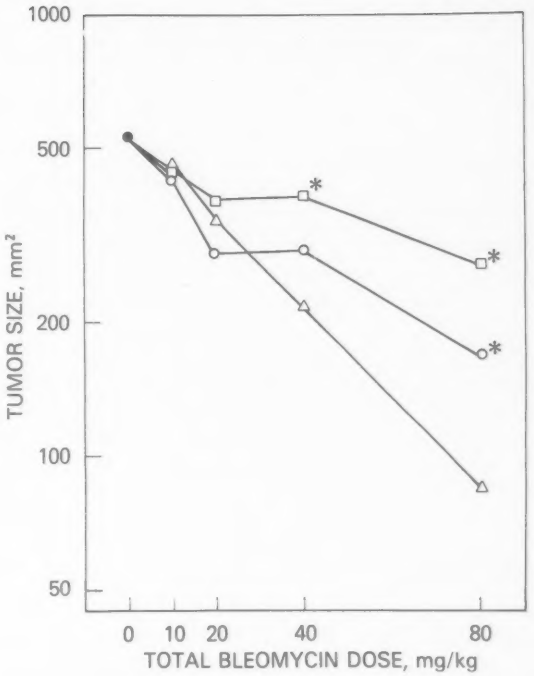

FIGURE 1.—Dose-response curve of bleomycin antitumor effect against Lewis lung carcinoma comparing 3 schedules of administration. These measurements were made on Day 15 after implantation, but are representative of differences which existed throughout the course of tumor growth. □——□ = injection sc twice weekly; ○——○ = injection sc 10 times weekly; △——△ = continuous sc infusion; ●— = control group; * = significantly different from continuous infusion, $P < 0.05$.

When nontumored animals were treated for 1 week with these three schedules of bleomycin, significant increases in lung collagen content were observed in both injection schedules at the highest nonlethal total dose, 40 mg/kg (fig 2) ($P < 0.05$, Student's t test). However, no such increase oc-

TABLE 1.—Effects of bleomycin dose schedule on survival of mice with Lewis lung carcinoma

Treatment	Survival in days*	% increase in lifespan
Saline controls	27.2 ± 7.5	—
Bleomycin (10 mg/kg twice weekly)	32.7 ± 5.1	20
Bleomycin (2 mg/kg 10 times weekly)	36.4 ± 4.8†	34†

*Mean ± SD; No. of animals = 10.
†$P < 0.05$ compared to saline controls.

curred in the group given this dose by continuous infusion. Another group of mice infused with a total dose of 40 mg/kg of bleomycin over 2 weeks, thus decreasing peak tissue levels but doubling duration of exposure, also showed normal mean lung hydroxyproline content (fig 2).

DISCUSSION

The effects of a particular dosage schedule upon the activity of an antineoplastic drug involve a complex interaction between the pharmacokinetic behavior of that drug, its actions on the various phases of the cell cycle, and the growth kinetics and drug sensitivity of individual tumors. Examples of the importance of proper drug scheduling in cancer treatment abound in the literature. Thus, intermittent, high-dose treatment with cyclophosphamide is much more effective than lower doses administered more frequently in the therapy for murine L1210 leukemia (14) and Burkitt's lymphoma in man (15). Goldin et al (16) found that the optimal schedule of methotrexate for L1210 leukemia varied, depending upon when in the course of the "disease" treatment was initiated. Cytosine arabinoside, which has a short plasma half-life and is cycle specific, is more effective when administered by continuous infusion or very frequent injections as opposed to intermittent injections of larger doses, both in mice and in human patients with leukemia (17,18).

Bleomycin also has a short plasma half-life when iv bolus injection is used, with rapid renal excretion in both mice and man (9,19). Furthermore, cells in the G_2 phase of the cell cycle appear to be most sensitive to the lethal effects of bleomycin, although this may vary with cell type (20). For human lymphoma cells in culture, increasing the duration of exposure to bleomycin from 4 to 35 hours (one cell cycle) dramatically increases cytotoxicity (8). It is not surprising, therefore, that continuous infusion of bleomycin in mice improved the therapy for Lewis lung carcinoma compared to the other two schedules examined.

Studies of bleomycin pharmacokinetics by infusion versus injection in man provide an intriguing possible additional mechanism for the differences in effects of these schedules (9,10). We have simulated bleomycin pharmacokinetics in man based on the empiric data and equations provided by those two papers (fig 3). Three schedules similar to those used in our mouse experiments were modeled: 10 units/m^2 by continuous iv infusion for 5 days, 5 units/m^2 q12hours by iv bolus, and 25 units/m^2 iv on Days 1 and 3, thus maintaining the same total drug dose (1.6 units of bleomycin ≈1 mg). Peak plasma levels are 0.16, 0.38, and 1.9 units/ml respectively (fig 3). It is striking that the elimination pharmacokinetics of bleomycin in man requires at least a two-compartment model, with a prolonged elimination phase beginning 12 hours after cessation of infusion. Both of the studies on which this model is based utilized the same radioimmunoassay of bleomycin, sensitive to 0.002 milliunit/ml, and this prolonged elimination phase was not observed in any patient treated with iv bolus injection (Strong J, personal communication).

Qualitatively, one explanation for the presence of a deep compartment which is seen after long-term infusion but not after rapid bolus injection might be a long distributive phase after the bolus, which does

TABLE 2.—Effects of 2 schedules of bleomycin injection on pulmonary toxicity

Treatment	No. of deaths/ No. of animals treated	Lung hydroxyproline* (μg/right lung)	% consolidation of lung parenchyma*
Saline controls	2/30	120.9 ± 3.0	0.3 ± 0.2 (2/28)
Bleomycin (2 mg/kg 10 times weekly × 4 wks)	8/32	179.0 ± 13.3†	8.3 ± 2.4† (13/23)
Bleomycin (10 mg/kg twice weekly × 4 wks)	1/32	151.6 ± 6.9†	7.7 ± 3.3† (12/30)

*Mean ± SEM; No. of animals = 30–32. Nos. in parentheses = Nos. of animals in each group that had > 1% consolidation of lung parenchyma upon morphometric analysis.
†Significantly different from control, $P < 0.05$.

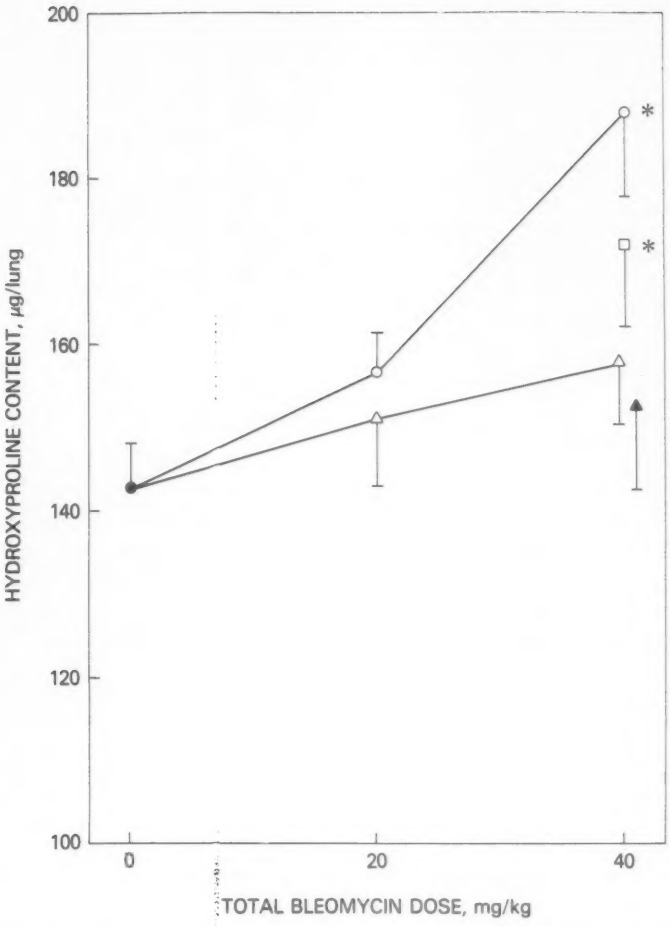

FIGURE 2.—Effects of various schedules and doses of bleomycin on pulmonary toxicity in nontumored animals, as measured by lung hydroxyproline content 10 wks after treatment. △——△ = continuous infusion for 1 wk; ▲——▲ = continuous infusion for 2 wks; ○——○ = injection 10 times weekly for 1 wk; □——□ = injection twice weekly for 1 wk; ●——● = saline controls; * = significantly different from controls, $P < 0.05$. Mean ± SEM; No. = 10.

not end until after sampling has stopped or the assay limit has been reached. The deep compartment may represent increased drug binding to DNA and/or a second site or mechanism for bleomycin activity. Thus, the apparent pharmacokinetic differences may account for the difference in the dose-response by schedule (fig 1). The plateau at intermediate doses of bleomycin given by bolus injection, but progressively increasing antitumor effect with increasing dose, may result from a second mode of drug action in the deep compartment after the primary mode in the central compartment has been maximally effective.

With linear pharmacokinetics, the pattern of drug exposure in the deep compartment would be analogous (though dampened) to that shown in figure 3 for the central compartment (multiple transients in drug concentration versus relatively constant level). In this case, if the therapeutic effect depends upon duration of exposure above a threshold level, the continuous-infusion schedule will be superior. However, more complex pharmacokinetic models might also be proposed, eg, saturable uptake into the deep compartment, which would increase the relative amount of drug in the deep compartment via the infusion schedule.

The decreased pulmonary toxicity associated with continuous infusion clearly indicates that the therapeutic effects of bleomycin may be selectively improved by dosage schedule, and this confirms the

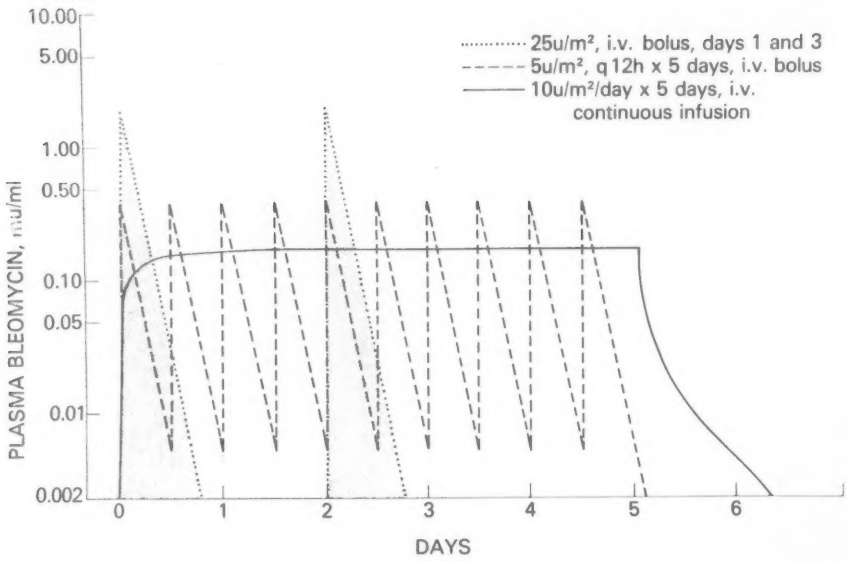

FIGURE 3.—Model of bleomycin pharmacokinetics in man comparing equivalent total doses of drug administered by 3 different schedules iv over a 5-day course of treatment. (1 mg of bleomycin = approximately 1.6 units of clinically formulated dose.)

clinical impression obtained in the treatment of testicular and cervical cancers (3,4). Pulmonary toxicity of bleomycin may exhibit a threshold phenomenon which does not occur below a certain plasma or tissue level of the drug, and the probability of exceeding this threshold is less when administered by infusion. However, the fact that multiple, low-dose injections produced appreciable pulmonary toxicity indicates that this threshold is quite low, and may even be exceeded by higher doses of continuous infusion (fig 3). Tissue clearance is a function of organ blood flow, drug uptake and efflux, and rates of drug metabolism, and these factors may vary considerably on a pharmacogenetic basis from individual to individual, so that there may be a wide range of thresholds for bleomycin lung toxicity in patients. That this is true even in inbred mouse strains is illustrated by the fact that at a 40-mg/kg total dose two of ten animals in the continuous-infusion group had lung collagen levels indicating pulmonary toxicity (> 164 μg of hydroxyproline per right lung), although the mean value for the entire group was normal. In each of the two intermittent injection groups, six of ten mice exceeded this level of hydroxyproline, and the mean for the group was significantly elevated compared to control or infusion groups.

Extrapolation of these conclusions from mouse to man will depend on careful clinical correlations which are not yet available. The data in animals suggest that the optimal dosage for bleomycin therapy is the maximum dose below the "threshold" for pulmonary toxicity. Peak plasma levels of bleomycin appear to be more important than duration of exposure for development of toxicity, and the peak plasma levels achieved by twice weekly injection would be 12 times greater than those achieved by the equivalent infusion (fig 3). In patients, there is as yet no means of predicting differences in sensitivity to bleomycin lung toxicity. However, determination of relatively nontoxic doses and durations of infusion should be done systematically in a randomized, controlled study in patients, which should also compare the therapeutic effectiveness of infusion versus injection.

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Phase I and Preliminary Phase II Study of Neocarzinostatin [1,2]

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SUMMARY

Neocarzinostatin is a protein antitumor antibiotic isolated from cultures of *Streptomyces carzinostaticus* var. *F41*. The drug has undergone extensive clinical trial in Japan, and has been reported active against a variety of human tumors. A phase I and preliminary phase II evaluation of the drug has been performed, using an iv bolus daily × 5 schedule. Ninety-six patients have been treated at doses from 500 to 2250 units/m²/day. Courses were repeated at 4-week intervals if allowed by bone marrow recovery. Dose-limiting toxicity was myelosuppression, which occurred late (median nadir, Day 27). Myelosuppression was more pronounced in patients who had received previous chemotherapy. In nine patients (9%) thrombocytopenia was prolonged (≥ 45 days) or irreversible. Acute administration of the drug was associated with rigors in approximately half the patients. Gastrointestinal side effects were mild. Three patients had a severe acute reaction resembling anaphylaxis. The maximally tolerated dose for this dose schedule is approximately 2250 units/m²/day. Antitumor activity has been seen in hepatoma and hematologic malignancies. Activity in lung and colorectal carcinoma appears limited with this dose schedule.

[Cancer Treat Rep 62:2019–2025, 1978]

Neocarzinostatin (NCS) is a polypeptide antibiotic isolated from the culture filtrate broth of *Streptomyces carzinostaticus*. It was first isolated in 1957 and was later purified by Ishida et al in Japan in 1964 (1). More recently, the structure of NCS has been elucidated by Meienhofer et al (2).

NCS is an acidic protein macromolecule of molecular weight 10,700 consisting of a single polypeptide chain of 109 amino acids cross-linked by two disulfide bridges. It is the first antibiotic protein for which the amino acid sequence has been determined, and it is the largest macromolecule of defined structure currently in use in clinical anticancer chemotherapy.

The precise mechanism of action of NCS has not been established. It has been shown to inhibit tritiated thymidine incorporation into DNA, to prevent cells from entering mitosis (3), and to cause single- and double-strand breaks in DNA (4). At high doses it has been shown to degrade DNA into free bases (5). In addition, it has been shown to interact with template DNA, lowering its template activity (6). Data from DON, Chinese hamster ovary, and HeLa cell systems indicate that the principal cytocidal effects of NCS occur in the G_2 phase of the cell cycle (7,8).

Recently, it has been shown that NCS covalently bound to agarose beads is capable of producing growth inhibition and inhibition of ^{3}H-TdR incorporation in CCRF-CEM cells (9), raising the possibility that the action of the drug may be mediated by cell membrane effects. In addition, NCS has been shown to inhibit surface immunoglobulin central capping induced by anti-immunoglobulin M antibody on Daudi cells (a cell line derived from Burkitt's tumor), an effect similar to those produced by agents affecting cellular microtubular systems such as colchicine and vinblastine (10,11).

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NCS has been found to be active against a variety of animal tumors including Sarcoma 180 (12), SN-36 leukemia (13), Ehrlich ascites carcinoma, and L1210 leukemia (14). In the latter in vivo system, there was no evidence of schedule dependency. Large animal toxicology studies have shown that the drug has a steep dose-toxicity curve. In studies performed by the National Cancer Institute, no toxic dose low could be found.

The highest nontoxic dose, the toxic dose high, and the lethal dose were 0.2, 0.4, and 0.8 mg/m^2 respectively in a single-dose schedule. The dose-limiting toxicity in large animal studies was interstitial nephritis while other toxic effects included anorexia, vomiting, fever, hemorrhagic diarrhea, and, at high doses, neutropenia and significant lymphopenia (15,16).

As a result of its significant antitumor activity in a variety of animal species, the drug has received extensive evaluation in Japan. More than 500 patients with a variety of malignancies have been treated. Most of the patients received doses of 0.04–0.06 mg/kg/day (~1.5–2.25 mg/m^2) × 4–20 consecutive days iv. Generally, repeat courses of therapy were administered at 4–10-day intervals. Toxic effects were reportedly mild and included anorexia (21%), nausea and vomiting (11%), fever (14%), skin rash (4%), and occasional episodes of hypotension and anaphylaxis. Myelotoxicity was manifested by neutropenia and thrombocytopenia which were reportedly uncommon with an overall incidence of 25% and 14% respectively. Possible renal toxicity was reported in only two patients. Antitumor activity was reported for pancreatic and gastric carcinomas and acute leukemia (15).

As a result of the drug's unique structure, the reported antitumor activity, and the lack of significant myelotoxicity, phase I studies were initiated in the US.

MATERIALS AND METHODS

NCS was provided by the National Cancer Institute, Bethesda, Md, and was obtained from Kayaku Antibiotics Research Co, Ltd, Tokyo, Japan. The drug was supplied in 2-ml vials containing 2000 units of NCS in sodium acetate-acetic acid buffer (0.015 M, pH 5.0). For purposes of comparison to Japanese drug regimens, 1400 units is considered equivalent to 1 mg.

The drug was administered by a rapid 1–2-minute iv injection or a 15–20-minute rapid infusion. When the drug was administered by a rapid infusion it was dissolved in 200 ml of 5% dextrose in water.

Patients were treated at the Sidney Farber Cancer Institute, Boston, Mass. Patients were eligible for the phase I study if they had a histologically confirmed metastatic malignancy for which there was no currently available therapy of known benefit or if they had failed previous appropriate chemotherapy regimens. Other eligibility criteria included no previous radiotherapy or chemotherapy within the preceding 3–4 weeks; a hematocrit of > 30%, a wbc count of > 3000/mm^3, and a platelet count of > 100,000/mm^3; a serum creatinine of < 2.0 mg/100 ml and/or a creatinine clearance of > 60 ml/minute; and normal liver function tests unless the abnormalities were secondary to hepatic metastases. All patients were informed of the nature of the study, and a signed informed consent was obtained prior to entry in the program.

Pretreatment evaluation included a complete history and physical examination with documentation of metastatic disease and objective tumor measurements when possible, laboratory studies including a cbc (differential, reticulocyte, and platelet counts), urinalysis, and liver function tests (SGOT, alkaline phosphatase, total proteins, albumin, and total and direct serum bilirubin). Renal function was measured by BUN, creatinine, and creatinine clearance in most patients; other biochemical studies included glucose, LDH, calcium, phosphorus, and uric acid. In addition, a chest roentgenogram and electrocardiogram were obtained in all patients.

After the initiation of therapy patients had a cbc and platelet count weekly; urinalysis and repeat liver and renal function tests were obtained every 2–3 weeks and prior to repeat treatment.

Repeat physical examinations with tumor measurements were performed at 3-week intervals. All pretreatment parameters were repeated prior to removal of a patient from the study.

Eighty-eight patients with a variety of malignancies were entered in the phase I study. Eighty patients were evaluable for toxicity. NCS was administered iv daily × 5 consecutive days. The dose escalation scheme is presented in table 1. The initial dose was 500 units/m^2, which represented approximately one third of the lower dose level employed in the Japanese studies. The plan was to rapidly escalate to a dose of 1500 units/m^2 if significant toxicity was not encountered. It was anticipated that repeat courses could be administered at 14–21-day intervals from the beginning of therapy, but because of the development of significant delayed myelosuppression repeat courses were generally administered at 28–35-day intervals. Eight subsequent patients were entered for phase II evaluation.

Cumulative myelotoxicity was defined as the appearance of significantly increased myelosuppression after re-administration of the same dose of

TABLE 1.—Dose escalation

Dose* (units/m²/day)	Study design (No. of evaluable patients entered)	No. of evaluable courses
500	3	3
1000	3	3
1500	14	14
2000	30	46
2250	38	60

*Schedule: iv bolus daily × 5 q28days.

TABLE 3.—Acute side effects: incidence per course

Dose (units/m²/day × 5)	No. of immediate reactions/total No. of courses	No. of cases of anorexia and nausea and vomiting/total No. of courses
1500	4/17 (24%)	3/17 (18%)
2000	11/46 (24%)	8/46 (17%)
2250	26/62 (42%)	20/62 (32%)

chemotherapy and/or a similar degree of myelosuppression after a reduced dose of chemotherapy. Leukopenia was defined as a wbc count of < 3000/mm³; thrombocytopenia was defined as a platelet count of < 100,000/mm³.

Complete response was defined as the complete disappearance of clinically detectable disease; partial response was defined as a > 50% shrinkage in the product of the largest perpendicular diameters of measurable disease.

RESULTS

Toxicity

Ninety patients were evaluable for acute toxicity and 82 patients were evaluable for chronic toxicity.

The eight patients unevaluable for the latter died < 28 days after drug administration. Pertinent patient characteristics are presented in table 2.

No toxicity was seen in patients treated with doses of 500 and 1000 units/m² respectively. Therefore, further toxicity data relate to patients treated with 1500–2250 units/m². Acute toxic effects included the development of an intense rigor which occurred within 30–60 minutes of injection and lasted from 30 to 60 minutes. The rigor generally occurred only on Day 1 of therapy, although occasionally it was seen on the subsequent days of drug administration. There was a tendency for this toxicity to be dose-related, with doses ranging from 1500 to 2250 units/m², although these differences were not statistically significant (table 3). The immediate reaction could be prevented in most patients by pretreatment with 50 mg of diphenhydramine iv prior to drug administration. A severe, immediate, anaphylactoid-type reaction consisting of flushing, hypotension, and seizures was seen in three patients (3%). The reaction was terminated by iv epinephrine and hydrocortisone in all patients. One patient was re-treated after such an episode with hydrocortisone administered prior to NCS administration. The anaphylactoid-like reaction did not recur. Gastrointestinal toxicity consisted primarily of anorexia with occasional mixed nausea and/or vomiting. This toxicity did not appear to be dose-related and was not dose-limiting.

TABLE 2.—Patient characteristics

	No. of patients (%)
No. of patients entered	96
Male/female	53/43
Median age	52
Range	6–86
Prior therapy	
Radiation therapy	61 (64)
Chemotherapy	72 (75)
Single-agent	17 (18)
Multiple-agent	49 (51)
Investigational	34 (35)

Severe, somewhat unpredictable myelotoxicity was the most significant toxicity encountered in patients receiving > 1500 units/m² (76 patients). The median wbc and platelet counts are shown for all patients and courses in table 4.

Although there appears to be a tendency for thrombocytopenia to be dose-related, this observation probably results from the drug's tendency to exhibit cumulative toxicity with repeat courses (*vide infra*). It should be noted that severe thrombocytopenia (< 20,000 cells/mm³) was encountered in some patients at all dose levels > 1500 units/m².

An analysis of the degree and timing of myelosuppression is presented in table 5. When myelosuppression occurred it tended to be moderate to severe and delayed. Considering all patients, thrombocytopenia (46%) was twice as common as leukopenia (22%). Neither the intensity nor the duration of myelotoxicity appeared to be dose-related. Leukopenia generally occurred prior to thrombocytopenia, but both effects were delayed when they occurred.

An analysis was performed to assess the effect of prior chemotherapy on myelotoxicity (table 6). Since

TABLE 4.—Myelosuppression

Dose (units/m²/day × 5)	No. of patients	No. of courses	Wbc nadir (× 10³/mm³)		Platelet nadir (× 10³/mm³)	
			Median	Range	Median	Range
1500	14	14	5.3	1.2–8.7	182	16–359
2000	30	46	4.4	0.7–10.2	116	11–290
2250	38	60	4.1	0.8–9.2	102	10–575

TABLE 5.—Degree and timing of myelosuppression

Dose (units/m²/day × 5)	No. of patients/total No. evaluable	No. of courses/total No. evaluable	Nadir (median)	
			Cells × 10³/mm³	Day
Wbc count ≤ 3 × 10³/mm³				
1500	2/14	2/14	1.2	20
2000	5/30	5/46	2.0	20
2250	9/38	9/60	1.8	20
Platelet count ≤ 100 × 10³/mm³				
1500	3/14	3/14	42	27
2000	15/30	21/46	60	28
2250	18/38	18/60	41	25

TABLE 6.—Effect of prior chemotherapy: 1st course only

Dose (units/m²/day × 5)	Prior chemotherapy*		No prior chemotherapy*	
	Wbc count ≤ 3 × 10³/mm³	Platelet count ≤ 100 × 10³/mm³	Wbc count ≤ 100 × 10³/mm³	Platelet count ≤ 100 × 10³/mm³
1500	2/11	3/11	0/3	0/3
2000	3/19	15/19	0/11	0/11
2250	6/26	13/26	1/6	4/6

* No. of patients with toxicity/total No. evaluable.

NCS has demonstrated significant cumulative toxicity in some patients, the analysis was performed for the first course of therapy only. At doses of 1500 and 2000 units/m², both leukopenia and thrombocytopenia were significantly more common in previously treated patients. No previously untreated patient experienced myelotoxicity at these dose levels. A 25% dose escalation from 2000 to 2250 units/m² produced a significant increase in thrombocytopenia in previously untreated patients: none of 11 patients at the 2000-unit dose compared to four of six patients at the 2250-unit dose.

More than one course of therapy was administered to 15 patients at doses of 2000 and 2250 units/m². Eight of 15 (53%) patients experienced cumulative myelosuppression, predominantly thrombocytopenia. When cumulative toxicity was encountered it tended to be protracted, with recovery to a platelet count of > 100,000/mm³ taking as long as 2–3 months in some patients.

Protracted thrombocytopenia generally occurred in patients with prior therapy, although it was also encountered in previously untreated patients receiving multiple courses of therapy.

No hepatic or renal toxicity or alopecia was encountered in this study. Extravasation of the drug led to severe cutaneous ulcerations in two patients who ultimately required skin grafting to close the defect. One patient died from pulmonary insufficiency after developing diffuse progressive pulmonary infiltrates on chest roentgenogram. Autopsies showed histopathologic changes of an

interstitial pneumonitis and small pulmonary venous thromboses. The etiology of this lesion and its relationship to NCS have not been determined.

Evaluation of Response

Patients who showed objective progression of disease after one course of therapy were considered nonresponders; otherwise, two courses of therapy were required for evaluation of response.

Of the 96 patients entered in this phase I–II study, 38 are unevaluable for response. Eighteen patients received one course of drug and were then withdrawn from protocol due to unacceptable toxicity, usually myelosuppression. Ten patients died within 4 weeks after receiving the drug, usually from progressive tumor, and were judged unevaluable. Six patients received nonmyelosuppressive doses during the early phase I study. Two patients had nonmeasurable disease, and two patients withdrew from the study for personal reasons.

Activity in colorectal carcinoma appears limited, with none of 14 patients responding. In contrast, NCS appears to possess definite antitumor activity in hepatoma.

Three of three patients with hepatoma showed partial responses that were evidenced by a decrease in hepatic size and an improvement in liver scan in the first patient, a decrease in a rectal mass palpable on examination in the second patient, and a decrease in hepatic size and shrinkage of multiple pulmonary lesions seen on chest roentgenogram in the third patient, with some pulmonary lesions undergoing complete regression. One of the 15 responses was noted in lung cancer (anaplastic non-oat cell). One of six evaluable patients with sarcoma and one of six evaluable patients with melanoma showed a partial response. However, these were of short duration.

A patient with mycosis fungoides showed transient partial regression of her skin lesions after one course of therapy. Extensive prior therapy precluded further treatment with NCS. A second patient with lymphosarcoma with extensive bone marrow involvement had several regressions of tumor, with intervening relapses when drug was withheld due to myelosuppression.

Four children with acute lymphoblastic leukemia were available for response. One child developed an M_1 marrow on Day 14 following the first course of NCS. However, the patient remained persistently thrombocytopenic, received no further therapy, and relapsed at 10 weeks. One adult patient had multiple relapses with acute myelogenous leukemia and showed clearing of peripheral myeloblasts but no marrow improvement.

DISCUSSION

NCS is the first of the "macromolecular" chemotherapeutic agents to reach trial in the US. These agents (NCS, macromomycin, mitamalcin, and others) are small proteins isolated from culture filtrates of various micro-organisms belonging to the genus *Streptomyces*, which show potent antitumor activity in animal systems.

Experimental evidence has been obtained that two of these agents (NCS and macromomycin) may exert their cytocidal effect through interaction with the membrane of the cell, without direct cellular entry (9,17). Thus, this group of protein antibiotics represents a new class of chemotherapeutic agents of unique chemical structure possessing a possibly unique mechanism of action.

The present study employed NCS at a schedule and at doses similar to previously reported studies from Japan (15). Despite the limited myelosuppression seen with NCS in the initial Japanese studies, NCS has proven to be a potent myelotoxic agent in this phase I–II study. Myelosuppression, particularly thrombocytopenia, was clearly the dose-limiting toxicity. The minimum toxic dose was 1500 units/m²/day × 5, and the maximum tolerated dose was 2250 units/m²/day × 5.

Prior chemotherapy appeared to be a significant risk factor predisposing to myelosuppression. The data presented in table 6 indicate that the myelosuppression associated with NCS administration occurred to a greater extent at lower doses in previously treated patients than in previously untreated patients. The data also suggest a very steep dose-toxicity relationship, even in previously untreated patients. As mentioned above, a steep dose-toxicity relationship was also noted in large animal toxicology experiments.

The thrombocytopenia induced by NCS occasionally occurred in an erratic manner, in that serious thrombocytopenia occurred at all dose levels greater than or equal to the minimal toxic dose (1500 units/m²). Onset of serious thrombocytopenia (< 50,000 platelets/mm³) could occur in the absence of previous chemotherapy, marrow involvement with tumor, or altered hepatic or renal function. When thrombocytopenia did occur, it tended to be delayed, sometimes protracted, and cumulative with repeat doses. This occurrence of severe, protracted thrombocytopenia after NCS administration was seen in approximately 10% of patients treated in these protocols.

Bone marrow aspirates and biopsies were performed on four patients with protracted thrombocytopenia. The bone marrow examinations were clearly hypoplastic in three. The fourth patient had

TABLE 7.—Evaluation of response

Disease	No. of patients—		Response		Duration of response (mos)
	Entered	Evaluable	Partial	None	
Gastrointestinal					
Colon/rectum	19	14	0	14	—
Hepatoma	4	3	3	0	5,7,5+
Gallbladder	2	1	0	1	—
Pancreas	2	1	0	1	—
Genitourinary					
Bladder	2	2	0	2	—
Hypernephroma	5	1	0	1	—
Prostate	1	0	—	—	—
Ovary	1	0	—	—	—
Cervix	1	0	—	—	—
Hematologic					
Acute lymphocytic leukemia	5	4	1	3	—
Acute myelogenous leukemia	1	1	0	1	—
Lymphoma	3	2	2	0	1,10
Other					
Lung	24	15	1	14	8
Sarcoma	10	6	1	5	1
Melanoma	7	6	1	5	1
Breast	4	0	—	—	—
Head and neck	1	0	—	—	—
Cylindroma	1	1	0	1	—
Unknown	3	1	0	0	—

multiple serial bone marrow examinations, each showing normal to mildly decreased overall cellularity and megakaryocyte markers. Radioisotopic platelet survival studies in the latter patient were within normal limits. The thrombocytopenia associated with NCS administration most probably results from decreased platelet production or release.

Concerning other chronic toxic effects noted in this study, no significant renal, hepatic, or cardiac toxicity occurred. In addition, the etiology of the interstitial pneumonitis mentioned above has not been established. The patient's lung tissue was examined for the presence of immune complexes; none were detected. The drug can cause severe cutaneous ulceration when extravasation occurs.

NCS can cause an acute reaction manifested by a severe rigor soon after drug administration. As noted, this generally occurs on the first day of drug administration, although it can occur on subsequent days. Anaphylactoid reactions were noted in three patients, each of whom responded to aggressive supportive care. Therefore, NCS should only be administered in a setting in which immediate physician availability or surveillance is assured.

Ohnuma et al (18) have reported the results of a phase I study which employed NCS administered iv as a 2-hour infusion or a continuous infusion for 120 hours. The dose-limiting toxicity of the 2-hour infusion was reached at 3000–4000 units/m^2 and consisted of a severe immediate reaction including rigor, fever, hypotension, respiratory distress, and tachycardia in three of five patients.

All patients responded to iv corticosteroids, acetaminophen, and diphenhydramine and recovered within 1–2 hours. At the maximal dose employed in our study, 2250 units/m^2, the immediate reactions were less severe, consisting predominantly of a rigor. The severity of the reaction described by Ohnuma et al may be a dose-related phenomenon, which would have occurred with further escalation of the dose in our patients. These preliminary data on the continuous infusion of the drug indicate that the maximally tolerated dose of NCS is 4500 units/m^2/day for solid tumor patients and 6000 units/m^2/day × 5 for patients with acute leukemia. The dose-limiting toxicity of the continuous infusion was myelosuppression, rather than the acute reaction, also indicating that the latter may be a result of peak blood levels associated with the rapid iv administration of NCS.

Based upon our data it appears that an appropriate starting dose for phase II studies is 1500 units/m^2/day × 5 for previously treated patients and 2250 units/m^2/day × 5 for previously untreated patients when the drug is administered by rapid iv injection or infusion. A course of therapy should be repeated

every 28–35 days. Although these doses may not produce significant myelosuppression in most of the patients, myelosuppression, when it occurs, may be severe and protracted. Dose escalations in subsequent courses should be used with caution since over half of the patients exhibit cumulative myelotoxicity with repeated courses.

Objective responses were seen in the diseases noted in table 7. Phase II data are necessary to elucidate the activity of NCS in the various solid tumors, particularly hepatoma. The most appropriate schedule of drug administration for patients with acute leukemia must be determined. Conceivably, a continuous infusion would be optimal considering the rapid half-life of NCS (19) after bolus administration and its reported G_2 phase specificity.

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cis-Dichlorodiammineplatinum(II) and Adriamycin Treatment of Advanced Ovarian Cancer [1,2]

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SUMMARY

Treatment with a combination of adriamycin (ADM) and *cis*-dichlorodiammineplatinum(II) (DDP) has been evaluated in 24 patients with advanced ovarian cancer. Twenty patients had received prior chemotherapy and/or radiation therapy. Objective remissions were seen in ten patients. The usual toxic manifestations of ADM and DDP were observed. It is concluded that the combination is effective therapy for ovarian cancer. Further investigation of this combination versus single agents is warranted.

[Cancer Treat Rep 62:2027–2030, 1978]

cis-Dichlorodiammineplatinum(II) (DDP) and adriamycin (ADM) have been shown to be active as single agents for the treatment of both previously untreated and previously treated patients with advanced ovarian cancer. The response to ADM alone is variously reported as 16%–45% (1–5). The response to DDP alone is 26%–31% (6,7) but the duration of response is short. As a third-line chemotherapy, it is ineffective (8). Vogl et al (9) introduced combination therapy with DDP and ADM. One of five patients with ovarian cancer responded to this regimen. Bruckner et al (10,11) further evaluated this combination in patients with advanced ovarian cancer. They reported a 70% overall response rate (28% complete response [CR] and 41% partial response [PR] in 43 women who had not received prior chemotherapy; a 40% overall response was observed in 60 previously treated patients. This combination has been extended to a cooperative study by the Cancer and Leukemia Group B. This group experience with the combination of DDP and ADM included 25 patients with advanced ovarian cancer, 20 of whom had failed to respond to prior chemotherapy and/or radiotherapy.

MATERIALS AND METHODS

All patients had histologic proof of ovarian cancer of epithelial origin and had stage III or IV disease at the time of diagnosis. All patients evaluated for tumor response had measurable disease. All patients had a life expectancy of at least 8 weeks. Written informed consent was obtained from all patients. Eight Cancer and Leukemia Group B institutions participated in the study (*see* footnote 3).

Patients with limited cardiac reserve who were judged to be unable to tolerate fluid load were excluded from the study. Also excluded were patients with a serum creatinine > 1.5 mg/dl, creatinine clearance < 50 ml/minute, bilirubin > 1.5 mg/dl, SGOT or SGPT 50% greater than normal, and wbc count < 4000/μl or platelet count < 100,000/μl.

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[3]This study was performed for the Cancer and Leukemia Group B by the following participating institutions: University of Minnesota Hospitals, Minneapolis (*K. E. Briscoe* and *B. J. Kennedy*, CA–16450); Cornell University Medical College, New York, NY (*M. W. Pasmantier*, CA–07968); Mt. Sinai School of Medicine, New York, NY (*T. Ohnuma*, CA–04457 and CA–16118); Columbia-Presbyterian Medical Center, New York, NY (CA–12011); Dartmouth Medical School, Hanover, NH (CA–04326); Inselspital, Bern, Switzerland; Long Island Jewish-Hillside Medical Center, New Hyde Park, NY (CA–11028); and Wilmington Medical Center, Del (CA–05462).

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Patients who had received prior treatment with ADM or DDP were also disqualified from entry.

ADM was obtained commercially from Adria Laboratories, Inc (Wilmington, Del) and was reconstituted in the standard manner. DDP, manufactured by Ben Venue Laboratories, Inc (Bedford, Ohio), was obtained from the Division of Cancer Treatment of the National Cancer Institute (Bethesda, Md); it was refrigerated until just prior to administration when it was reconstituted with 10 ml/vial of normal saline. DDP was protected from light exposure and was administered within 1 hour of reconstitution. Both drugs were administered at a dose of 60 mg/m^2.

Patients were hydrated overnight with at least 1.5 liters of 5% dextrose and one-half normal saline infused at 200 ml/hour. The infusion rate was then increased as necessary to achieve a baseline urine output of at least 100 ml/hour for 4 consecutive hours without the use of diuretics. At this point, 60 mg/m^2 of ADM was diluted in 75 ml of normal saline and infused over 30 minutes. This was followed by 12.5 g of mannitol given as an iv bolus and 60 mg/m^2 of DDP reconstituted in 5% dextrose and one-half normal saline and administered as two separate aliquots (30 mg/m^2), each over 1 hour. Supplemental iv fluids and mannitol infusions (0–5 g/hour) were utilized as necessary to maintain a urine output of 100 ml/hour during the DDP infusion and for 6 hours thereafter. Serum electrolytes were monitored and managed appropriately.

Treatment was repeated every 3–4 weeks. The ADM dose was modified if severe myelosuppression occurred (table 1). DDP was modified if the creatinine clearance dropped to < 50 ml/minute or if the serum creatinine rose to > 1.5 mg/dl. Combined therapy was continued to a maximum ADM dose of 500 mg/m^2.

To be evaluable for tumor response patients had to receive at least two courses of therapy and had to be observed for at least 3 weeks following therapy.

TABLE 1.—Dose modification*

Wbc count (cells/μl)	Platelet count (platelets/μl)	ADM dose
	Day 1	
≥ 4000	≥ 100,000	100%
3000–3999	50,000–99,999	50%
< 3000	< 50,000	Defer for 1 wk
	Nadir	
1500–3500	50,000–99,999	100%
< 1500	< 50,000	50%

*If creatinine is > 1.5 mg/dl omit DDP only; resume with regular cycle when creatinine is ≤ 1.5 mg/dl.

CR was defined as 100% regression of all measurable tumor persisting for at least 3 months. PR was defined as a 50%–99% regression in the sum of the products of the longest perpendicular diameters of all measurable lesions persisting for a minimum of 3 weeks without simultaneous increase in the size of any lesion or the appearance of any new lesions. Patients who did not meet these criteria were considered treatment failures. Response intervals were measured from the initiation of therapy to definite progression or recurrence.

RESULTS

Thirty patients were entered in the study between October 1976 and September 1977. Six patients were not included in the evaluation because of major protocol violations (reduced initial dose in five and inadequate peripheral blood cell counts in one).

Of the 24 evaluable patients, ten were responders (four CRs and six PRs), for an overall response rate of 42% (table 2). The duration of responses ranged from 11 to 58 weeks; the median duration was 24½ weeks. Three of the four complete responders had not received any prior systemic chemotherapy.

Fourteen patients did not meet our criteria for demonstrating tumor response and are therefore considered to be treatment failures. Of these patients, five received only one course of therapy (two patients refused further therapy because of protracted vomiting, one patient was lost to followup, and two patients died of progressive disease before their second course of treatment). Four patients had progressive disease after two courses of treatment. The remaining five patients demonstrated stabilization of disease for varying periods of time (10–23 weeks).

The definitions used in assessing toxicity are listed in table 3. The regimen was moderately toxic. All patients experienced profound, and in some instances, persistent nausea and vomiting. All patients developed alopecia, anemia was significant in 12 patients, thrombocytopenia necessitated a dose reduction or a delay in the administration of ADM in 11 patients, and leukopenia was associated with serious infection in one patient. Seven patients had a decrease in renal function. The dose of DDP was modified or omitted for one course in three of these patients with subsequent improvement in renal function; in no instance did this directly affect survival. There were no instances of clinical hearing loss or congestive heart failure.

TABLE 2.—Characteristics of the 10 responding patients

Patient No.	Age (yrs)	Prior therapy	Extent of disease	No. of treatments to best response	Best response	Response duration (wks)*
1	39	None	Abdominal	6	CR	26
2	45	Systemic chemotherapy†	Abdominal	3	CR	30
3	46	Abdominal irradiation	Abdominal	2	CR	30
4	55	None	Abdominal	4	CR	24+
5	63	Systemic chemotherapy	Lung, liver, and pelvis	2	PR	15½
6	49	Systemic chemotherapy	Abdominal	2	PR	15½
7	76	Systemic chemotherapy	Pelvis and pleura	2	PR	58
8	49	Abdominal irradiation and systemic chemotherapy	Pelvis and abdominal	4	PR	24½
9	55	Peritoneal lavage with chromic phosphate, intrapleural chemotherapy, and systemic chemotherapy	Pleural and pericardial	2	PR	18½
10	65	Systemic chemotherapy	Abdominal and supraclavicular nodes	1	PR	11

*From initiation of therapy.
†Systemic chemotherapy = alkylating agents or 5-fluorouracil.

TABLE 3.—Major toxic effects secondary to DDP and ADM (20 patients)*

Complication	No. of patients		
	None	Mild	Severe
Anemia	8	4 (decrease of Hgb 1–3 g/dl)	8 (decrease of Hgb > 3 g/dl)
Leukopenia	7	4 (1500–3000 cells/μl)	9 (< 1500 cells/μl)
Thrombocytopenia	11	5 (50,000–99,999 platelets/μl)	4 (< 50,000 platelets/μl)
Nephrotoxicity	13	14 (serum creatinine 1.6–1.7 mg/dl, decrease in creatinine clearance < 30 ml/min from baseline)	3 (serum creatinine > 1.8 mg/dl, decrease in creatinine clearance < 30 ml/min from baseline)

*Excludes 4 early-loss patients for whom no toxicity data are available.

CONCLUSIONS

Treatment with a combination of DDP (60 mg/m²) and ADM (60 mg/m²) every 3–4 weeks resulted in significant tumor regression (four CRs and six PRs) in ten of 24 women with advanced ovarian cancer. The response duration ranged from 11 to 58 weeks. Prior treatment did not adversely affect the duration of response.

This cooperative study has confirmed the efficacy, but does not demonstrate the superiority, of the combination of DDP and ADM over either agent alone. Comparative phase III trials are warranted.

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Combined Modality Treatment of Burkitt's Lymphoma [1]

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In 1976, the Pediatric Oncology Branch (POB), Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, reported the results of a combined modality treatment regimen (POB protocol 74–0) in 15 patients with Burkitt's lymphoma (1). Treatment included three courses of induction chemotherapy (cyclophosphamide, vincristine, and methotrexate), prophylactic intrathecal methotrexate, and postinduction whole-abdomen radiotherapy for patients with abdominal tumors. There were two early deaths, 13 complete responses (CR), eight relapses, and five long-term survivors in this initial trial.

Following completion of this study, a second treatment regimen (POB protocol 75–6) introduced two major changes: (*a*) prednisolone was added to the induction combination, and (*b*) the third induction course was replaced by a double dose of cyclophosphamide. The rationale for these changes was based on the addition of another lympholytic agent in high dose, and incorporation of an "early intensification" strategy with high-dose cyclophosphamide at a time when the tumor burden was minimal. This report compares the results of these two sequential treatment protocols.

PATIENTS AND METHODS

Protocol 75–6 was initiated in May 1975 and was closed to further entries in June 1977. Twenty-two eligible patients (with histologic proof of Burkitt's lymphoma and no prior therapy) were treated. Details of clinical evaluation and staging have been previously described (1,2), and staging criteria are shown in table 1. Details of the treatment regimen appear in table 2, and, for purposes of comparison, the preceding protocol (74–0) is also summarized.

TABLE 1.—Clinical staging of Burkitt's lymphoma

Stage	Extent of tumor
A	Single extra-abdominal site
B	Multiple extra-abdominal sites
C	Intra-abdominal tumor
D	Intra-abdominal tumor with involvement of multiple extra-abdominal sites
AR	Stage C but with > 90% of tumor surgically resected

Patients who relapsed received re-induction chemotherapy with the same 75–6 protocol; if CRs occurred, patients were recycled through the entire protocol, omitting abdominal irradiation. Incomplete or transient responders entered an intensive chemotherapy regimen consisting of BCNU, cytosine arabinoside, cyclophosphamide, and 6-thioguanine (BACT), followed in some cases by an infusion of autologous bone marrow previously harvested and cryopreserved during remission (3).

Patients with central nervous system (CNS) involvement (malignant lymphoma cells in the cerebrospinal fluid with or without cranial neuropathy) received 2500 rads of whole-brain irradiation and intrathecal chemotherapy consisting of cytosine arabinoside (30 mg/m^2 intrathecally, Days 1–3) and methotrexate (15 mg/m^2 intrathecally, Day 4), repeated every 2 weeks for three courses. These drugs and sequence were chosen because: (*a*) both agents are active in Burkitt's lymphoma; (*b*) a 4-day regimen would expose tumor cells to drug for at least four potential doublings; and (*c*) methotrexate would follow cytosine arabinoside to avoid holding up cells at the G_1/S interphase of the cell cycle. Systemic chemotherapy (POB protocol 75–6) was reinitiated even if CNS involvement was the only manifestation of relapse.

RESULTS

Patient characteristics are shown in table 3. All 22 patients achieved a CR, as judged by criteria described in previous reports (1,2).

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TABLE 2.—Protocols 74-0 and 75-6

Protocol No.	Drug	Dose (mg/m^2)	Route	Schedule* (Day)
74-0	Cyclophosphamide	1000	iv	1
	Vincristine	1.4	iv	1
	Methotrexate	15	Intrathecal	1,4
	Methotrexate	15	iv	2,3
75-6†	Cyclophosphamide	1000	iv	1
	Vincristine	1.4	iv	1
	Methotrexate	12.5	Intrathecal	2,5
	Methotrexate	12.5	iv	1,3,4
	Prednisolone	1000	iv	1–5

*3 chemotherapy courses were repeated at 2-wk intervals (ie, Days 1, 15, and 29); courses were delayed until the total wbc count was > $4500/mm^3$.

†Instead of a third induction course, patients receive cyclophosphamide (1200 mg/m^2 iv) on each of 2 successive days. Methotrexate (12.5 mg/m^2) was given intrathecally on the first day of double-dose cyclophosphamide, followed by citrovorum factor rescue (5 mg im q6h × 4). 2 wks following the completion of chemotherapy, all patients with stages C, D, and AR received 2100 rads of whole-abdominal radiation over 18 elapsed days.

Relapse occurred in nine (41%) patients. Their duration of remission was 3 months (range, 2–6), and the minimum followup was 16 months. Initial sites of relapse were: abdomen, six patients; CNS, two patients; and testes, one patient. All relapses but one (patient 6) developed in patients with stage C or D tumor, and occurred within 6 months of presentation. Five of the six patients with intra-abdominal relapse had completed abdominal irradiation; the sixth patient (No. 15) had an intra-abdominal recurrence before irradiation could be initiated.

Treatment of relapse resulted in transient CRs in seven patients with systemic relapse. Two additional patients (Nos. 16 and 19) who developed isolated CNS relapse have had sustained second remissions. Four patients with drug-resistant relapse underwent intensive BACT chemotherapy, and two (patients 7 and 13) have prolonged remissions.

Seventeen (77%) patients are living and have been free of disease for > 1 year. This group is composed of 13 patients with sustained initial remission and two each with prolonged second or third remissions.

The total wbc counts after each course fell to a median nadir of $1000/mm^3$ at 10 days, with recovery to > $4500/mm^3$ at 15 days from the start of therapy. In four patients profound neutropenia (wbc count < $1000/mm^3$) was accompanied by fever and bacteremia. Four additional patients developed fevers of unknown origin following chemotherapy. Two patients had grand mal seizures. One seizure followed the instillation of methotrexate intrathecally. The other was attributed to profound hyponatremia (serum sodium < 116 mEq/liter) resulting from overhydration and possible cyclophosphamide renal toxicity. All patients who underwent abdominal irradiation suffered varying degrees of nausea and anorexia.

Factors associated with long-term survival are age at onset of disease and initial disease stage. As in previous reports, advanced age is associated with a poorer prognosis. While the population was evenly distributed around a median age of 12 years, four of the five deaths occurred in patients ≥ 13 years of age. Furthermore, four of five deaths occurred in patients with stage C or D tumor. These trends do not achieve statistical significance because of the relatively small number of patients and a low mortality rate; they are consistent, however, with previous reports (1,2).

DISCUSSION

This report confirms the relatively good prognosis of patients with Burkitt's lymphoma who are managed with aggressive chemotherapy. It is not possible to evaluate the respective roles of steroid or double-dose cyclophosphamide in the remission-induction regimen. Since remission induction and relapse rates are similar to those found with the preceding protocol 74–0, these changes are unlikely to have affected the clinical course in a substantive way.

Intrathecal methotrexate as used in protocols 74–0 and 75–6 does not avert CNS relapse entirely, since two patients in this series relapsed solely in this site. However, intrathecal chemotherapy, supplemented with whole-brain irradiation, may be curative in the setting of CNS involvement as the

TABLE 3.—Patient characteristics (all patients achieved CR)

Patient No.	Age	Sex	Stage	Disease sites*	Prior surgery	Remission duration (mos from treatment)	Relapse site*	Treatment of relapse†	Survival (mos from treatment)
1	9	M	A	Tonsil	Tonsillectomy	16+			16+
2	12	M	B	Nodes, NASO, BM, bone, CNP	—	20+			20+
3	4	M	AR	Ileocecal	Tumor resection	34+			34+
4	9	M	AR	Ileum, MES	Tumor resection	31+			31+
5	39	F	AR	Ileum, MES	Tumor resection	25+			25+
6	12	M	AR	Ileocecal	Tumor resection	3	ABD	75-6, BACT, no BMA	5
7	10	F	C	Bowel, MES		4	Pelvis	75-6, BACT-BMA	34+
8	21	F	C	ABD		2	ABD	75-6	4
9	18	M	C	ABD	Hemicolectomy	3	ABD	75-6, BACT-BMA	9
10	34	M	C	ABD	Regrowth after previous resection	16+			16+
11	3	M	D	CERV, KID	Nephrectomy	29+			29+
12	8	M	D	ABD, pelvis, pleura	—	28+			28+
13	12	M	D	NASO, L-S	—	4	Testis, BM, RETRO	75-6, BACT-BMA	24+
14	39	F	D	CERV, CNP, RETRO, BM	—	20+			20+
15	29	F	D	Ovary, MES, breast	—	22+			22+
16	24	M	D	Facial bone, MED, RETRO	—	2	CNP, CSF	75-6, CRT/COMP, ITRx	19+
17	9	M	D	ABD, pleura, MED	—	19+			19+
18	7	M	D	ABD, pleura	—	18+			18+
19	6	M	D	CERV, ABD	—	2	CNP, CSF	75-6, CRT/ITRx	18+
20	14	F	D	ABD, ovary, pleura	—	6	ABD	75-6	8
21	16	M	D	ABD, pleura	—	4	ABD, BM	75-6	5
22	19	M	D	BM, CSF, skin, bone, L-S	—	15+			15+

*NASO = nasopharynx, BM = bone marrow, CNP = cranial nerve palsy, MES = mesentery, ABD = abdomen, CERV = cervical nodes, KID = kidney, L-S = liver-spleen, RETRO = retroperitoneal, MED = mediastinum, CSF = cerebrospinal fluid.

†CRT = cranial radiation therapy; BACT (*see* text); BMA = bone marrow autograft; COMP = cyclophosphamide, vincristine, methotrexate, and prednisone; ITRx = intrathecal chemotherapy.

TABLE 4.—Comparison of collaborative results of protocols 74-0 and 75-6 (*from* ref 2)

Protocol No.	No. of— Patients treated	Early deaths	CRs/total evaluable patients	Relapses (% CR)	Deaths (% CR)
74-0					
National Cancer Institute	15	2	13/13 (100%)	8 (61%)	7 (54%)
Non-National Cancer Institute	14	0	14/14 (100%)	5 (36%)	5 (36%)
Totals	29	2	27/27 (100%)	13 (48%)	12 (44%)
75-6					
National Cancer Institute	22	0	22/22 (100%)	9 (41%)	5 (23%)
Non-National Cancer Institute	17	2	13/15 (86%)	6 (46%)	6 (46%)
Totals	39	2	35/37 (95%)	15 (43%)	11 (31%)

sole relapse site, since both patients continue in long second remissions.

The role of postchemotherapy abdominal radiation is speculative. Five of the six abdominal relapses occurred among 19 patients whose abdomens were irradiated. Since this regimen is often tolerated poorly and recurrence in the irradiated field has been noted, the notion of "sterilizing" residual tumor with this dose of radiation can be seriously questioned (2).

For purposes of comparison, table 4 reviews results of the present National Cancer Institute series compared to patients followed collaboratively in this protocol and in its predecessor, protocol 74–0 (table 2). This collaborative study was reported in detail elsewhere (2), but patient followup is now substantially longer and more data from protocol 75–6 are available. Several points emerge. The CR rate is equally high with both regimens and overall relapse rates are similar (48% for protocol 74–0 and 43% for protocol 75–6). Both groups are comparable with respect to age and stage, and differences in mortality (44% for protocol 74–0 and 31% for protocol 75–6) are not statistically significant. The trend towards improvement may reflect increasingly successful management of CNS and systemic relapse in patients treated at the National Cancer Institute. When fully developed, intensive chemotherapy and bone marrow autografting may find a role in primary therapy for patients at high risk of relapse (4).

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Attempted Drug-Tumor Sensitivity Matching Using Diffusion Chamber-Cultivated Cells [1,2]

Barbara H. Mason [3] and Bruce F. Cain [4,5,*]

SUMMARY

Cells from L1210 leukemia, B16 melanoma, Lewis lung tumor, and a C3H spontaneous mammary tumor line (C27) have been cultivated in millipore diffusion chambers implanted ip in mice. After chamber implantation, there is an initial drop in tumor cell number followed by a period of exponential growth and a later plateau phase in which cell numbers remain reasonably constant. Cells in plateau phase remain viable to dye-exclusion techniques and on reimplantation into animals provide tumors at the site of inoculation. Single oral doses of chlorambucil, melphalan, and cyclophosphamide (0.8 × LD10), as well as ip administered single doses of cytosine arabinoside or 6-mercaptopurine (q3h × 8; 0.7–1.0 × LD10), provided statistically significant ($P < 0.05$) cell-kills of exponentially growing, chamber-cultured C27 cells. Ip administered 6-thioguanine (q3h × 8; 0.7–1.0 × LD10) failed to provide a significant cell-kill. Simultaneous administration of 6-mercaptopurine and cytosine arabinoside provided a significantly greater tumor cell-kill than either agent alone, without additional host lethality. The combination of 6-thioguanine and cytosine arabinoside did not provide a greater effect than that provided by the latter agent alone. Of those drug treatments successful in the diffusion chamber experiments, only the two providing the greatest reduction in tumor cell numbers (cyclophosphamide and a combination of cytosine arabinoside plus 6-mercaptopurine) provided significant ($P < 0.05$) extensions of mean lifespan when administered to animals bearing the C27 tumor. The formidable logistics associated with arranging sufficient numbers of chamber-implanted mice for an adequately controlled study of any tumor sample preclude application of the present methods to attempted clinical drug-tumor sensitivity matching. Chamber-cultivated tumor slices or fragments, in which characteristic histologic or morphologic features remain, might permit drug-sensitivity matching with cell-cycle stage nonspecific agents, but ranking of responses must then be by subjective methods.

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Earlier, an attempt was made to develop a drug-tumor sensitivity matching procedure employing millipore diffusion chamber-cultivated tumor specimens (1). Cells from both a C3H spontaneous mammary tumor line and L1210 leukemia were cultivated in such chambers implanted ip in allogeneic mice. Following drug treatment of the host animals, effects on the tumor cells were monitored cytologically. For the L1210 leukemia, at least, the chemotherapeutic sensitivity of this tumor, progressing in its syngeneic host, could be predicted from the results of such diffusion chamber experiments. Wider application of drug-tumor matching by this

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technique, particularly when seeking evidence of the effectiveness of S-phase specific agents or the superiority of drug combinations, requires a greater knowledge of the kinetic behavior of the chamber-cultured cells and effective quantitation of the tumor cell-kill provided by agents.

MATERIALS AND METHODS

Animal and Tumor Strains

Both tumor lines and necessary inbred animal strains were originally obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Mice used were bred in the animal facility of this laboratory. Tumors were passaged by completely standard protocols (2). Shortly after receipt, cell suspensions (10^7 cells/ml) were prepared from these tumors in TC-199 medium supplemented with 10% fetal calf serum (FCS) and dimethylsulfoxide (10%), frozen by slow cooling (2°C/minute), and stored in liquid nitrogen.

The mammary tumor used (C27) is the 27th in a series of such lines derived from the spontaneous tumors arising in retired female breeders of the C3H/He mouse strain. After six passages at 3-week intervals, implanting 5×10^6 tumor cells im in the right thigh of C3H/He mice, a cell suspension of this tumor was frozen as above. This mammary tumor may be classified as having a medium growth rate (3) from the observed mean survival times of C3H/He mice following implantation of cell suspensions (5×10^6 im, 42–54 days; 5×10^6 sc, 29–37 days). This particular tumor was chosen for the following studies because of its relative refractoriness to most presently employed agents.

All tumors are maintained in the laboratory by serial transfer for no longer than 3 months, and are then replaced with samples of the depoted material from frozen storage.

Tumor Cell Suspensions

Single-cell suspensions of the L1210 leukemia were obtained directly from the peritoneal cavity of DBA_2 carrier mice that had initially received ip tumor implants. Following cell counting by hemocytometer, suitable dilution with TC-199 medium containing 10% FCS provided suspensions of necessary concentration (5×10^6 cells/ml).

Lewis lung adenocarcinomas (1.5–2.0 cm in diameter), removed from sc implantation sites, were suspended in cold (2°C–5°C) TC-199 medium and teased between two scalpel blades until dispersed. Following gravity filtration through two thicknesses of 100-mesh nylon, cells were collected by centrifugation ($200 \times g$ for 3 minutes) and suspended in cold TC-199 medium containing 10% FCS (5 ml). Following hemocytometer counting, dilutions were made to provide a concentration of 10^6 cells/ml suitable for the loading of diffusion chambers.

To obtain single-cell suspensions of the B16 melanoma and C27 tumor, a freshly dissected tumor (> 1.5 cm in diameter) was chopped finely with curved-pointed iris scissors while immersed in a few milliliters of freshly thawed TC-199 (2°C–5°C) in the center of a watchglass. The sludge of cells was decanted periodically and replaced with fresh cold medium; this process continued as long as substantial numbers of cells continued to be dislodged. The resulting suspension was applied to a sintered glass funnel (8-cm diameter plate; porosity No. 1) which had been prewetted with chilled medium. The suspension was filtered under unit gravity and the funnel was rinsed through with fresh medium. The filtrate was collected in ice-cooled tubes and the cells were collected by centrifugation at $200 \times g$ for 3 minutes. The resulting pellets were suspended in TC-199 containing 10% FCS to provide a final concentration of 10^6 viable cells/ml (yield, about 10^7–10^8 cells/g of tumor). While providing a poor recovery of viable tumor cells, the above filtration method is excellent for obtaining single-cell suspensions.[6]

Viability, in all cases, was assessed by dye exclusion employing a total concentration of 0.2% trypan blue in the suspensions employed for hemocytometry.

Diffusion Chambers

Methods used for construction, sterilization, filling, sealing, animal anesthesia, and surgical implantation were previously described (1); the chambers contained 0.175 ml of tumor cell suspension. All of the chamber recipients were C3H/He mice. Loading density for the solid tumors employed was 10^6 cells/ml.

Cells were dislodged from diffusion chambers for counting purposes by minor modification of published methods (4–6). Immediately after harvesting, the chambers were lightly sponged with a tissue moistened with Ringer-Locke solution, and then gently agitated in a freshly prepared 0.5% solution of pronase (B grade, Commonwealth Serum Laboratories, Parkvill, Australia) in TC-199 (3 ml/chamber) for 1 hour at ambient temperature. The chambers were then shaken gently for 5 minutes in an

[6]We are grateful to Drs. P. and E. Stephenson, School of Biological Sciences, University of Sydney, Australia, for details of this technique.

EDTA solution (EDTA, 0.675 g; NaCl, 2.0 g; KCl, 0.05 g; $Na_2HPO_4 \cdot 2H_2O$, 0.37 g; KH_2PO_4, 0.05 g; and glucose, 0.05 g in H_2O [250 ml], pH adjusted to 7.2 with 1 N NaOH). After checking the integrity of the enclosing membranes, the membrane opposite to the one on which the cells were originally settled (1) was pierced with a Pasteur pipette and the contents were transferred to a tared bottle. The chamber was rinsed with three changes of EDTA solution. Viable cells in aliquots of the totalled washings were counted and, from the total weight of the latter, assuming 1 g is equivalent to 1 ml, the cellular contents of the chambers were derived. Results of counts have been expressed as cells per ml of chamber content. The empty chambers were fixed in 10% formol-saline and the membranes were stained and examined as before (1). Use of either the pronase or EDTA treatment alone was markedly less successful in dislodging cells and disrupting cellular clumps than was the sequential use of both solutions.

Agent Details

Following preliminary range-finding experiments, LD10 values for the alkylating agents employed were obtained using groups of ten C3H/He mice (20–25 g) at each dose level. Dose levels were separated by 0.05 log/dose intervals and together spanned the range expected to provide from 0 to 100% lethality. Animals were observed daily for 40 days. Percent probit mortalities and the logarithms of the corresponding doses were correlated employing a computer program based on published procedures (7). Derived LD10 values, in mg/kg for single oral doses with 95% confidence limits in parentheses, were: chlorambucil, 42.1 (34.3–45.3); melphalan, 17.48 (12.5–20.3); and cyclophosphamide, 322 (231–374). The LD10 for ip administered cyclophosphamide was 302 (222–360) mg/kg. Dose levels of the remaining agents employed were based on previous laboratory experience with these agents in C3H/He mice. Dose levels of these agents 0.18 log interval higher than those employed invariably provided a percentage of toxic deaths; the levels employed should then represent 0.7–1.0 of the LD10.

In oral dosing, by gavage, mice were starved overnight (15–16 hours) and an agent was administered in a volume of 0.025 ml/g of animal body weight. Doses for ip injection were contained in a volume of 0.01 ml/g of animal weight.

Antitumor Assays

A suspension of 5×10^6 C27 tumor cells in a volume of 0.1 ml was usually implanted im into the thigh of the right leg of C3H/He mice, but for certain experiments the suspension was implanted sc above the right axilla. Tumor progression was monitored twice weekly by vernier caliper measurement of maximum thigh diameter in the case of im implants (8,9) or two dimensions of the tumor at right angles when implantation was sc. In the latter case, tumor volumes were calculated using the formula for a prolate sphere, $4/3\ \pi\ ab^2$, where a and b are the major and minor semiaxes respectively (10). Animal deaths were monitored twice daily.

RESULTS

Diffusion Chamber Investigations

Changes in tumor cell densities in chambers with time showed an initial lag phase, a stage of rapid growth which, within the limits of accuracy of the counting procedures, appeared essentially exponential in character, and a final plateau phase, during which cell densities remained reasonably static (fig 1). The doubling times observed with the different tumors during exponential phase varied quite markedly (L1210 leukemia, 13 hours; C27 tumor, 18 hours; Lewis lung tumor, 30 hours; and B16 melanoma, 42 hours). Tumor cells in plateau phase were viable based on trypan blue exclusion, and provided tumors when reinoculated into syngeneic animals.

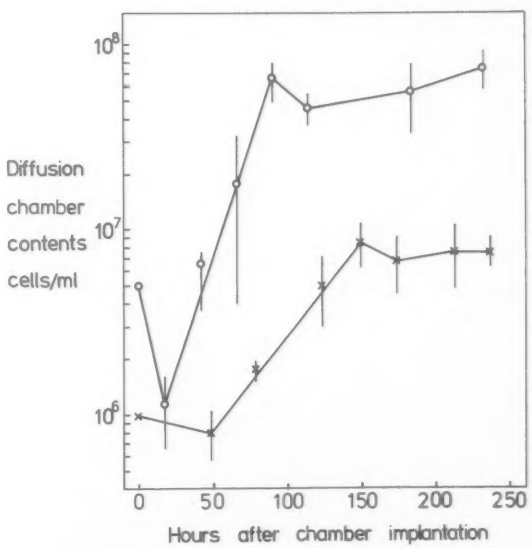

FIGURE 1.—Growth patterns for cells of the Lewis lung carcinoma (×) and the L1210 leukemia (O) cultivated in millipore diffusion chambers and implanted ip in mice. Cell counts are the mean from 4 chambers and SDs are shown as vertical bars.

Thus, plateau-phase C27 tumor cells (5×10^6) implanted sc in C3H/He mice provided tumors at the inoculation site. When chambers containing B16 tumor cells were deliberately retained in animals for 1 month, the cells harvested at that time still actively excluded trypan blue dye and similarly provided tumors when implanted sc in C57BL/6 mice. After inoculation of plateau-phase L1210 cells (240 hours after implantation) into groups of DBA_2 mice, at 5×10^6 and 5×10^4 cells/mouse, the recipients died of advanced leukemia and had mean lifespans of 7.0 and 9.3 days respectively; the lifespans were not significantly different from what would be expected by inoculation of similar numbers of cells on routine in vivo passage.

When the aim of diffusion chamber studies is to demonstrate the chemotherapeutic sensitivity of the cultivated tumor cell population, such growth patterns (fig 1) impose clear experimental strictures. To obtain the effects of drugs on an actively progressing tumor population, agents should not be administered to the animal host until there is evidence that the cultivated cells have progressed from lag phase and are in exponential growth. This requires withholding drug treatment for a period of 18–60 hours, depending on the tumor examined, after the chambers have been implanted in the animals. In the experiments reported which used the C27 tumor, drug treatment was initiated 70 hours after implantation. Cell densities in sample chambers (withdrawn at the time of drug treatment) and those of untreated controls when these were later harvested (together with the earlier measured doubling times), provided an additional check that cell growth was exponential when agents were administered.

As described previously (1), the consequences of drug treatment of chamber-cultivated cells appear relatively slowly. In line with previous findings, a significant difference in cell numbers between treated and control chambers did not occur until 3 days after chlorambucil was administered orally. Such differences in numbers were still evident 24 hours later, which was 168 hours after chamber implantation. However, at this late stage, cells in untreated controls were approaching the plateau phase of growth while surviving tumor cells in treated chambers were proliferating exponentially. Differences in cell numbers between treated and control chambers became attenuated with time; there is a quite limited time during which drug effects can be satisfactorily demonstrated. In subsequent experiments with C27 tumor cells, chambers were sampled both 3 and 4 days after single-dose treatment. In all cases, the differences between treated and control cell densities, as provided by T/C values (table 1) were greater on the third day following drug treatment than on the fourth day. In drug-sensitivity assays under these conditions (drug treatment 70 hours after implantation; harvesting 3 and 4 days later), the alkylating agents chlorambucil, melphalan, and cyclophosphamide all provided statistically significant levels of tumor cell-kill (table 1).

In preliminary experiments (not reported), C27 cell populations in exponential growth cultivated in diffusion chambers appeared sensitive to the S-phase specific agent cytosine arabinoside. Since high combination toxicity indices have been found for correctly scheduled combinations of cytosine arabinoside and 6-thioguanine (11,12) and therapeutic synergism is often observed with these agents (11,13,14), it was of interest to examine the inhibitory effects of such a combination in relation to that provided by cytosine arabinoside alone. In a set of experiments performed at one time (table 2), cytosine arabinoside provided a significant cell-kill while 6-thioguanine did not, and a combination of the two provided an intermediate effect. In direct contrast, in a further diffusion chamber experiment, 6-mercaptopurine and cytosine arabinoside each singly provided significant levels of tumor cell-kill (at the 5% level), and proved even more effective when combined (table 2).

Tumor Inhibitory Studies

To investigate the effectiveness of the agents employed against the C27 tumor, and the possible interrelationship between the cell-kills observed in diffusion chamber experiments and in vivo responsiveness, inhibitory assays were carried out employing animals with im implanted tumor (table 3, figs 2 and 3). To be of clinical value the results of diffusion chamber drug-matching techniques must be able to select those agents capable of at least significantly extending the lifespan of cancer patients. The same evaluation criterion should then be employed in experimental studies. Despite many suggestive delays in tumor growth (figs 2 and 3), only two drug treatments, those employing oral cyclophosphamide and a combination of cytosine arabinoside and 6-mercaptopurine, provided significant increases in lifespan in animal studies at better than the 0.05 percentile probability level (table 3).

While a single, large, oral dose of cyclophosphamide furnished a significant increase in host lifespan, there is a relatively long recovery time following treatment before an additional dose of this agent can be safely administered. Because of the reduction in cell numbers, and as a consequence of the Gompertzian growth characteristics of solid

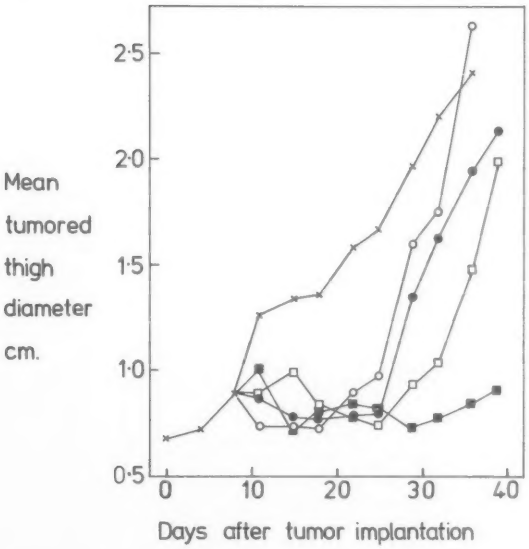

FIGURE 2.—In vivo chemotherapeutic sensitivity of im implanted C27 mammary tumor. Animals were implanted with 5×10^6 tumor cells im. Treatment groups, 10 mice each, were administered 0.8 of the single-dose LD10 on Day 8. There were 30 animals in the control group (×). ○ = oral chlorambucil; ● = oral melphalan; ■ = oral cyclophosphamide; □ = ip cyclophosphamide.

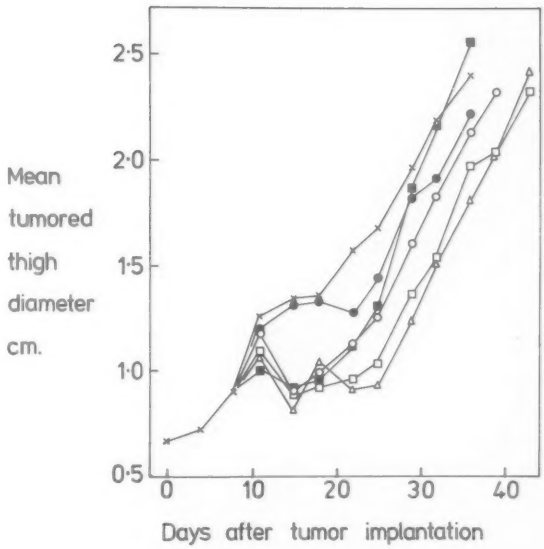

FIGURE 3.—In vivo chemotherapeutic sensitivity of im implanted C27 mammary tumor. Figs 2 and 3 comprise sections of a single experiment. Controls (×) are defined in fig 2. Agents were administered ip using q3h × 8 schedules on Days 8 and 12; doses were as detailed in table 3. ○ = cytosine arabinoside; ● = 6-thioguanine; □ = 6-mercaptopurine; ■ = cytosine arabinoside + 6-thioguanine; △ = cytosine arabinoside + 6-mercaptopurine.

TABLE 1.—Chemotherapeutic sensitivity of C27 mammary tumor cells cultivated in diffusion chambers

Drug treatment of host mice*	Chamber harvest time (hrs after drug treatment)	Chamber contents† (cells/ml)	*P* value‡	Ratio of cells in treated/ control mice (%)
Control	72	$8.6 (\pm 3.4) \times 10^5$		
Oral chlorambucil	72	$1.6 (\pm 0.5) \times 10^5$	0.01	19
Control	96	$1.0 (\pm 0.2) \times 10^6$		
Oral chlorambucil	96	$3.6 (\pm 2.6) \times 10^5$	0.01	36
Control	74	$8.0 (\pm 2.3) \times 10^5$		
Oral melphalan	74	$2.45 (\pm 1.0) \times 10^5$	0.01	31
Control	98	$6.6 (\pm 2.3) \times 10^5$		
Oral melphalan	98	$3.3 (\pm 2.2) \times 10^5$	0.01	50
Control	74	$8.0 (\pm 2.3) \times 10^5$		
Ip cyclophosphamide	74	$9.1 (\pm 1.7) \times 10^4$	0.001	11
Control	98	$6.9 (\pm 2.3) \times 10^5$		
Ip cyclophosphamide	98	$1.1 (\pm 0.4) \times 10^5$	0.01	16

*All drugs were administered once at 0.8 of the single-dose LD10.
†Cell counts are the mean from 4 chambers with the SDs in parentheses.
‡Student's t test; probability that there is a significant difference between treated and control cell numbers.

TABLE 2.—Scheduled drug treatment of exponentially growing C27 tumor cells

Drug treatment of host mice	Dose* (mg/kg/ injection)	Chamber contents† (cells/ml)	*P* value‡	Ratio of cells in treated/ control mice (%)
	Experiment A			
Control	—	1.1 (± 0.5) × 10^6		
6-Thioguanine	0.4	9.4 (± 2.3) × 10^5	0.6	85
Cytosine arabinoside	13	1.9 (± 0.9) × 10^5	0.02	17
6-Thioguanine + cytosine arabinoside	0.4 13	4.9 (± 1.3) × 10^5	0.1	45
	Experiment B			
Control	—	8.3 (± 2.9) × 10^5		
Cytosine arabinoside	13	3.7 (± 1.2) × 10^5§	0.05	44
6-Mercaptopurine	6.7	3.3 (± 1.1) × 10^5¶	0.02	40
Cytosine arabinoside + 6-mercaptopurine	13 6.7	1.3 (± 0.4) × 10^5§,¶	0.005	16

*All agents were administered ip in a q3h × 8 schedule.

†Cell counts are the mean from 4 chambers with the SDs in parentheses. Chambers were harvested 73 hrs after the start of drug treatment.

‡Probability that there is a significant difference between treated and control cell numbers.

§Probability that there is a significant difference between these 2 figures is 0.01.

¶Significant difference between these 2 figures at the 0.02 probability level.

TABLE 3.—In vivo chemotherapeutic response of im implanted* C27 mammary tumor

Agent	Dose (mg/kg/ injection)	Route and schedule	Mean survival time† (days ± SD)	*P* value‡
Control	—	—	48.4 (± 12.7)	
Chlorambucil	34	Single dose orally, Day 8	51.8 (± 9.4)	0.4
Melphalan	14	Single dose orally, Day 8	53.1 (± 6.8)	0.2
Cyclophosphamide	242	Single dose ip, Day 8	49.8 (± 8.5)§	
Cyclophosphamide	258	Single dose orally, Day 8	65.1 (± 14.3)§	0.01
Cytosine arabinoside	13	q3h × 8 ip, Days 8 and 12	50.2 (± 4.7)	
6-Thioguanine	0.4	q3h × 8 ip, Days 8 and 12	50.9 (± 14.3)	
6-Mercaptopurine	6.7	q3h × 8 ip, Days 8 and 12	53.4 (± 5.9)	0.1
Cytosine arabinoside + 6-thioguanine	13 0.4	q3h × 8 ip, Days 8 and 12	53.6 (± 6.4)	0.1
Cytosine arabinoside + 6-mercaptopurine	13 6.7	q3h × 8 ip, Days 8 and 12	55.5 (± 7.8)	0.05

*5 × 10^6 tumor cells were implanted im in the right hind leg on Day 0. There were 10 mice/treatment group and 30 control animals.

†Calculated from the 1st day of drug treatment.

‡Significance of the difference in lifespans of treated and control groups employing the Student's t test.

§Difference between these 2 lifespans is significant at the 0.05 probability level.

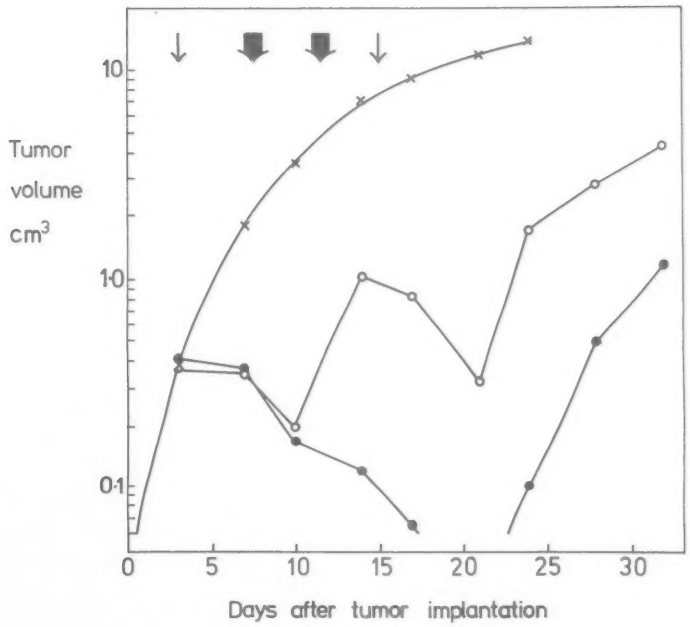

FIGURE 4.—Combination drug treatment of sc implanted C27 mammary tumor. There were 20 untreated control animals (×). 1 treated group of 10 mice received oral doses (258 mg/kg) of cyclophosphamide alone (○) at the times indicated by the narrow arrows. The remaining group (●) received cyclophosphamide as above and additionally q3h × 8 schedules of cytosine arabinoside + 6-mercaptopurine (9 and 4.5 mg/kg/injection respectively) at the times indicated by the broad arrows. Mean survival times in days, with SDs in parentheses, were: controls, 33.2 (± 8.3); cyclophosphamide alone, 50.7 (± 9.1); combination treatment group, 58.8 (± 8.6).

tumors, viable tumor cells remaining after cyclophosphamide treatment will multiply at greater rates than those prevailing at the time of initial treatment (15–17). To avoid such attenuation of initial tumor cell-kill, Schabel (16,17) has formulated the strategy of following doses of relatively cycle-phase non-specific drugs, such as cyclophosphamide, with courses of S-phase specific agents. There is usually an appreciably shorter necessary time before the S-phase specific drugs can be safely administered, and the higher growth fraction of the residual tumor cells augments the attainable levels of cell-kill with such agents. From the experiments reported it could be conjectured that, following an initial oral dose of cyclophosphamide, scheduled treatment with a combination of 6-mercaptopurine and cytosine arabinoside 4 and 8 days later might permit a further repeat dose of cyclophosphamide to be safely administered on Day 12. In an initial experiment employing sc implanted C27 tumor (so that tumor volume alone could be monitored at all times), and the dose levels used earlier, there were 30% early, presumably drug-related, deaths in the combination-treatment groups. Repetition employing reduced doses of the S-phase specific agents and an initial tumor size of 400 mg furnished the data shown in figure 4. Attempted multiple use of the same dose of cyclophosphamide employed, as a possible alternate treatment, on Days 3, 9, and 15 provided 70% toxic deaths. As judged from figure 4, the cyclic drug treatment is clearly the most effective of those examined and, although no animal was cured, such treatment would appear to be a prime candidate for eradicative trials when initial burden of this tumor is lower (eg, in a surgical adjuvant situation).

DISCUSSION

Oral cyclophosphamide appeared clearly superior to ip administered agent in extending the lifespan of animals bearing the C27 tumor when equal fractions of the measured LD10 doses were employed (table 3). This result could reflect: (*a*) the relatively broad confidence limits in the measured LD10 doses, resulting in the employment of doses which were not strictly equitoxic, or (*b*) the different rates of agent absorption and therefore activation, depending on the route of administration. The relative effectiveness of oral and ip administered cyclo-

phosphamide requires investigation using a range of dose levels.

Not all of the drug treatments providing statistically significant levels of tumor cell-kill in the diffusion chamber experiments could successfully inhibit growth of the parental tumor in animals (when an increase in lifespan was used as the assessment criterion). The two-drug treatments which provided the greatest reduction in cell numbers in the chamber experiments, oral cyclophosphamide and ip cytosine arabinoside plus 6-mercaptopurine, were those that furnished significant life extensions in the animal assays. This might suggest that of the order of a one-log kill must be observed in chamber experiments before significant inhibition of tumor growth in animals can be expected. Such sparse data must be viewed with considerable caution and we have already enumerated various biochemical, cytokinetic, and pharmacologic reasons why the results of diffusion chamber drug-matching may not be directly transferable to the usual tumor-host situation (1).

The results of the diffusion chamber trials did suggest that cytosine arabinoside and 6-mercaptopurine together can provide a greater cell-kill of C27 tumor cells than either agent alone, and this observation led to the development of a quite effective combination treatment (fig 4). This might suggest a role for diffusion chambers in screening possible drug combinations for use against those clinical cancers which appear relatively resistant to most agents, when these are employed singly. Unfortunately, the logistics of arranging such screening assays are quite formidable. When assessing drug effects by counting the cultivated tumor population, the relatively high standard deviation of those counts requires that multiple chambers be examined for each drug or drug combination assessed and at each time of harvest. From the growth pattern observed (fig 1), considerable preliminary investigations would have to be carried out, with each individual tumor, to determine when exponential growth commenced and the time at which plateau phase was reached. Until these details were available, drug sensitivities, particularly for S-phase specific agents, could not be easily examined. For the drug-matching step, the necessary harvest times to provide significant differences in cell numbers between treated and control chambers would have to be determined. If cellular doubling times of human tumor isolates growing in diffusion chambers were correspondingly longer than those seen with the animal solid tumors, then attempted drug-matching would be a lengthy procedure.

The divergent time scales of the substitute mouse host, and that of the cultivated tumor cells from a patient, pose critical problems. Treatment schedules of, for example, cytosine arabinoside must be arranged to the time scale of the cultivated tumor cells to ensure that a significant level of cell-kill will result. Drug doses to the mouse host must be arranged to provide concentration × time relationships comparable to those which would apply in the patient. It is not known what these doses should be or even if they would be tolerated by mice. The difficulty of arranging adequate groups of chamber-implanted animals to permit effective examination of the many factors influencing tumor cell-kill is a major deficiency of the method. Attempted drug-sensitivity matching of clinical samples by the cell-counting procedures employed would be impractical.

The added complication of the cellular heterogeneity of many clinical tumor samples, and the selection pressures exerted during preparation of adequate cell suspensions and the later lag-phase of growth, make it questionable that the contents of diffusion chambers could be confidently assumed to be an adequate representation of a patient's tumor. Further, the morphologic modulation of cells, when adapting to essentially two-dimensional growth on millipore membranes, and the later enzymatic steps necessary to dislodge these for counting purposes, make clear identification and therefore quantitation of tumor cells alone a formidable task. For practical drug-sensitivity matching of clinical tumor samples by diffusion chamber technology, an alternative approach to that employed here should be investigated. However, each of the variants so far examined appears to have its own individual set of deficiencies.

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Potential Anticancer Agents. XVIII. New Developments in the Benzoic Acid Nitrogen Mustards Area [1,2]

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SUMMARY

In order to obtain aromatic nitrogen mustards with improved therapeutic index against experimental neoplasms, > 75 new compounds were synthesized and studied. Their structure-activity relationship analysis led to the following conclusions: (*a*) the carboxylic group (especially when located in the *meta* position with respect to the nitrogen mustard group) exerts a favorable effect on the biologic properties of such compounds, probably by improving their transport characteristics; (*b*) a linear relationship was found between the chemical reactivity (expressed as alkylation rate, log k66) and toxicity (LD50) of 31 investigated compounds; and (*c*) the *ortho* effects also seem to be of importance in this area for a more accurate control of the nitrogen mustard activity. Other criteria (ie, log P, nucleophilicity of the target centers) involved in the rational design of aromatic nitrogen mustards are discussed. The design of new derivatives was oriented toward compounds which (*a*) were able to couple with selected proteins (ie, antibodies) leaving the cytotoxic moiety intact, and (*b*) were obtained by coupling of the 3-*N*,*N*-bis(2-chloroethyl)amino-4-methyl-benzoyl moiety with selected carriers (steroids, chromanones, etc) by way of an esteric bond.

[Cancer Treat Rep 62:2045–2054, 1978]

In the past the class of nitrogen mustards has furnished a great number of broad-spectrum antineoplastic compounds, some of which are being used clinically. Although a considerable part of the work done in this area was brought about on a random basis (1), attempts were made to find a rational grounds for such work (2–4) (ie, by using structure-activity relationships or exploitable differences between normal and malignant cells). From the large group of screened nitrogen mustards, compounds containing acidic functions (especially with carboxylic groups, ie, chlorambucil, isomeric phenylalanine mustards, high reactive nitrogen mustards, etc) received special attention, since the available data suggested that they might represent effective carriers for this cytotoxic moiety (4,5). This could partly be attributed to their increased hydrophilic character and also to a higher affinity for plasma proteins (3,6). Some excellent reviews substantiated this point of view (2–4,7).

The objective of our investigations over the last 10 years was to find better and more selective nitrogen mustards with acidic functions. This paper examines the data accumulated in our laboratory using this approach.

MATERIALS AND METHODS

The synthesis of most of the nitrogen mustards discussed in this paper was previously reported (8–11). Their purity was checked by elemental analysis and thin-layer chromatography. In each case the spectral data (ultraviolet, infrared, and nuclear magnetic resonance) were in good agreement with the proposed structures. The basicity of precursor

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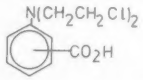
TABLE 1.—Isomeric nitrogen mustards derived from aminobenzoic acids



A (1-3)

No.	Compound	Hydrolysis rate, log k66 (s-1)	LD50 (mmols/kg body weight)	Antineoplastic activity: Jensen sarcoma (TGI%)	Antineoplastic activity: W256 carcinosarcoma (TGI%)	Antineoplastic activity: Ehrlich ascites carcinoma* (ILS%)
1	*o*-CO_2H	4.23	0.06	89	54	59
2	*m*-CO_2H	4.65	0.38	95	41	73
3	*p*-CO_2H	5.26	0.57	86	—	42

*The data for this system are reported in ref 36.

amines (analytically pure) was spectrometrically determined (12,13). The hydrolysis rates were measured according to Ross' (14) standard procedure ($Me_2CO:H_2O$ = 1:1, 66°C, 30 minutes). The alkylation rate was determined using the 4-(*p*-nitrobenzyl)pyridine (NBP) procedure (15). The partition coefficients (log P) of some of the compounds were measured in an octanol-water system. In some cases they were calculated (16,17). LD50 values were obtained using Wistar rats by the method described by Cornfield and Mantel (18).

The new compounds were tested against Jensen sarcoma, Walker 256 carcinosarcoma (W256), and L1210 leukemia. For the solid tumors, 0.1–0.2 LD50 was inoculated daily for 14 days in tumor-bearing rats. The treatment began 7 days after the tumor transplantation. The results were calculated according to the formula: TGI[5] = (1–T/C) × 100%.

The screening against L1210 was done according to the National Cancer Institute protocol guidelines and the data were expressed as ILS[6] = (T/C–1) × 100%.

Data on P388 leukemia and B16 melanoma were obtained from the National Cancer Institute, Bethesda, Md (for NSC-146171 and NSC-146172).

RESULTS AND DISCUSSION

It has been demonstrated that among a closely related series of nitrogen mustards a certain level of reactivity (3) is necessary for the compound to be an effective tumor growth inhibitor. On the other hand, high chemical reactivity is disadvantageous in designing compounds with selective toxicity against malignant cells (19). Of particular interest are those compounds which, in contrast with the normal behavior, couple a high antitumor activity with a relatively low chemical reactivity. For those compounds, particular mechanisms of action are probably involved. The isomeric nitrogen mustards of *o*-, *m*-, and *p*-aminobenzoic acids (***1–3***) represent such an interesting case (table 1) (4,5). A careful examination of their biologic properties reveals that: (*a*) these compounds divert from the above-mentioned correlation between hydrolysis rate and biologic activity, and (*b*) the *m*-substituted derivative possesses the best chemotherapeutic index in three tumor systems among the three isomers.

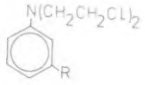
These observations prompted us to ascribe special transport properties (for the nitrogen mustard moiety) to the *m*-benzoic structure (and particularly to the carboxylic function) due both tc reduction of the reactivity of the nitrogen mustard group (by a –I effect) and also to the fact that the carboxylic group may be considered as increasing the possibilities of (ionic)interactions with serum proteins. It should be mentioned that besides their involvement in drug transport they could confer some protection against the hydrolysis of the alkylating moiety (20,21). Keeping this idea in mind, we designed, synthesized, and studied a new series of closely related aromatic nitrogen mustards in order to: (*a*) check the hypothesis concerning the importance of the carboxylic group, (*b*) exploit the previously described observations, (*c*) have a sufficient number of structurally related nitrogen mustards available for structure-activity analysis, and (*d*) find new compounds with a better therapeutic index.

Carboxylic Group Importance

In order to check this point, a series of *m*-substituted nitrogen mustards (B compounds, ***4–27***) were synthesized and the relationships between the nature of the substituents, hydrolysis rates, and biologic properties were investigated (table 2). The data thus obtained showed the following: (*a*) A

[5]TGI = tumor growth inhibition.
[6]ILS = increase in lifespan.

TABLE 2.—*m*-substituted nitrogen mustards



B/4-271

No.	R =	σm*	Hydrolysis rate, log k66†	LD50 (mmols/kg body weight)	Anticancer activity (TGI%) Jensen sarcoma	W256 carcino-sarcoma
2	CO_2H	0.35	4.65	0.38	95	—
4	Cl	0.37	4.55	—	0	—
5	I	0.35	4.68	—	35	—
6	NO_2	0.71	5.05	0.95	0	—
7	$NH_2 \cdot HCl$	—	4.54‡	0.18	58	—
8	CN	0.56	5.00	—	60	—
9	CO_2CH_3	0.32	4.46	0.52	93	40
10	$CO_2iC_3H_7$	0.32	4.49	0.90	99	54
11	$CO_2(CH_2)_2N(C_2H_5)_2 \cdot HCl$	—	4.73‡	0.72	92	70
12	COCl	—	—	0.53	85	40
13	$CONH_2$	0.26	4.36	0.38	87	78
14	$CONHCH_2CO_2H$	—	—	—	79	—
15	$CON(CH_3)_2$	0.21	4.36	0.17	48	—
16	$CON(iC_3H_7)_2$	—	4.38	0.43	0	—
17	$CONC_2H_4$§	—	—	1.74	60	—
18	$CONC_5H_{10}$¶	0.29	4.46	0.44	31	—
19	$CONC_4H_8O$**	—	—	0.75	85	58
20	$CONC_3HNS$††	0.36	4.57	0.87	93	60
21	$CONHC_6H_5$	0.21	4.30	0.41	33	—
22	$CONH(NO_2)C_6H_3CH_3(2',4')$	—	—	1.21	0	—
23	CONHNHCO	—	4.39	0.58	93	71
24	SO_2F	0.33	4.50	1.67	0	—
25	SO_2NH_2	0.46	4.82	0.34	37	—
26	$SO_2NHC_6H_4CO_2H(4')$	—	—	1.20	50	—
27	$SO_2OC_6H_5$	—	—	1.40	0	—

*σm = Hammett constants of *m*-substituents.
†Calculated from Ross test assuming a monomolecular kinetic.
‡Measured as hydrochlorides.
§Aziridinyl.
¶Piperidinyl.
**Morpholyl.
††Thiazolyl.

linear relationship can be obtained between hydrolysis rate and σm (Hammett).

$$\log k66 = 3.68\ (\pm\ 0.30) - 2.47\ (\pm\ 0.48)\ \sigma m$$
$$(n = 12;\ r = 0.90;\ s = 0.140)$$

(*b*) The favorable effect of the carboxylic group upon the anticancer activity was confirmed because compounds with nearly the same hydrolysis rate as 3-*N*,*N*-bis(2-chloroethyl)amino-benzoic acid (***2***), but with other substituents in the *meta* position (ie, I, Cl, SO_2F, etc), did not exhibit comparable lower antitumor activities. The moderate effectiveness of the nitrile derivative (***8***) may be accounted for by an "in vivo" hydrolysis (demonstrated in aromatic nitriles (22), which gave rise to the acid ***2***.

$$\text{Ar–CN} \xrightarrow{\quad} \text{Ar–CONH}_2 \xrightarrow{\quad} \text{Ar–COOH}$$
$$\textit{8} \qquad\qquad \textit{13} \qquad\qquad \textit{2}$$

Support for this sequence seems to be provided by the intermediary activity of the amide ***13***. (*c*) Esters ***9–11*** proved to be very effective against solid tumors (being generally superior to the amidic derivatives), probably because of the rapid in vivo enzymatic hydrolysis of such functions.

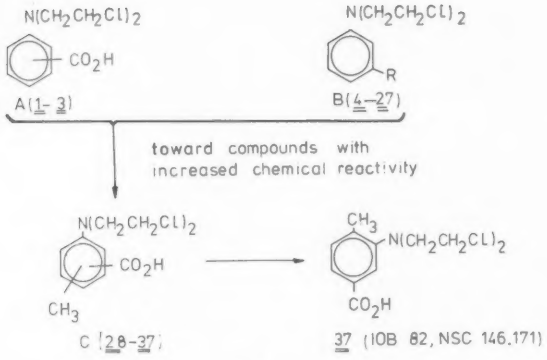


FIGURE 1

Nitrogen Mustards Derived From Isomeric Toluic Acid

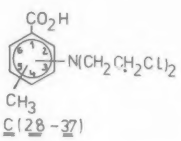
Since the carboxyl group induces a too-strong decrease in the chemical reactivity of the nitrogen mustard function (pka of aniline, 4.57; pka of *o*-, *m*-, and *p*-methylaminobenzoate, 2.32, 3.57, and 2.49 respectively [23]), we tried to restore it partially by introducing on the benzene nucleus a third electron-repelling substituent, which could increase the basicity of the nitrogen atom (pka of methyl aminotoluic esters being between 2.03 and 4.06, table 3). Thus, the ten isomeric nitrogen mustards of toluic acids were synthesized (C compounds, ***28–37***) fig 1, table 3).

All C compounds (***28–37***) exhibited strong antitumor properties, the most inhibitory being the 3-*N*,*N*-bis(2-chloroethyl)amino-4-methyl-benzoic acid (***37***, IOB-82, NSC-146171) (table 4), which had a pharmacologic behavior sufficiently promising for clinical trial (24). The clinical results were in some respects superior to those of other nitrogen mustards (ie, for head, neck, and tongue neoplasms in intra-arterial perfusions), mainly in view of the low leukopenic effect at therapeutic doses (25).

It is of interest to note that in compound ***37***, the nitrogen mustard is in the *meta* position with respect to the carboxyl function.

The correlation of biologic data with structural features of compounds C (***28–37***) led to the following conclusions: (*a*) The introduction of methyl group (+I effect) on the aromatic nucleus reasonably increased the chemical reactivity of the alkylating moiety and afforded very strong inhibitors of tumor growth. (*b*) The comparison of the basicity of unsubstituted amines (whose protonation is less sensitive to the steric effects of *ortho* substituents) and the hydrolysis rate of nitrogen mustards revealed a series of interesting *ortho* effects. The first effect is an abnormal increase of hydrolysis rate of *ortho*-methyl-substituted nitrogen mustards (eg, ***28***, ***33***, and ***37***). This effect may be partially accounted for by a steric hindrance of mesomerism (10,26). A similar but weaker effect is obtained also by an *ortho*-carboxyl substitution. A similar explanation may be advanced, but in this case the enhancement

TABLE 3.—Nitrogen mustards derived from isomeric toluic acids



C (28 - 37)

No.	Compound NM =	Compound $N(CH_2CH_2Cl)_2$	Basicity of precursor amine* (pka)	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antitumor activity (TGI%) Jensen sarcoma	Antitumor activity (TGI%) W256 carcino-sarcoma
28	$2CH_3$	3NM	3.50 ± 0.05	3.19	0.054	98	65
29	$2CH_3$	4NM	2.81 ± 0.03	4.72	0.209	79	55
30	$2CH_3$	5NM	4.06 ± 0.04	4.14	0.224	96	75
31	$2CH_3$	6NM†	2.90 ± 0.03	—	1.086	99	54
32	$3CH_3$	2NM	2.03 ± 0.03	3.65	0.075	90	50
33	$3CH_3$	4NM	2.18 ± 0.04	3.58	0.060	90	70
34	$3CH_3$	5NM	3.90 ± 0.04	4.44	0.190	59	40
35	$3CH_3$	6NM	2.54 ± 0.03	4.22	0.091	98	60
36	$4CH_3$	2NM	2.98 ± 0.04	4.08	0.091	100	42
37	$4CH_3$	3NM	3.34 ± 0.02	3.33	0.063	100	86

*Spectrophotometrically determined.
†Obtained as methyl esters.

TABLE 4.—Effectiveness of compound *37* against L1210 and P388 leukemias*

Schedule† (No. of doses)	Dose (mg/kg body weight)	Survival: No. of survivors/ No. treated	Survival: T‡ (days)	Survival: C§ (days)	Survival: T/C (%)
		L1210/BDF$_1$			
9 daily	12.5	6/6	16.4	9.3	176
	6.25	6/6	11.3	9.3	121
	3.12	6/6	10.1	9.3	108
	1.56	6/6	9.6	9.3	103
		P388/BDF$_1$			
9 daily	4.40	6/6	24.0	10.0	240
	3.00	6/6	21.0	10.0	210
	2.00	6/6	20.0	10.0	200

*Screening was done by the National Cancer Institute.
†Ip administration.
‡Leukemia-bearing animals.
§Controls.

of the basicity obtained by the hindrance of the p-π conjugation (due to the steric repulsion of the two adjacent bulky groups) is partially counteracted by the strong –M effect of the carboxyl function (10). This observation shows that attempts to deactivate aromatic nitrogen mustards by introducing bulky electron-attracting *ortho* substituents may lead to the opposite effect. Such *ortho* effects could be exploited in a more accurate control of the hydrolysis rate of aromatic nitrogen mustards.

Nitrogen Mustards Derived From 3-*N*,*N*-bis(2-chloroethyl)amino-4-methyl-benzoic Acid

Our attempts to improve the pharmacologic properties of 3-*N*,*N*-bis(2-chloroethyl)amino-4-methylbenzoic acid by synthesis of new compounds are shown in figures 2 and 3 and led to a group of derivatives, nitrogen mustards D (*38*–*44*) (table 5), in which the *ortho*-methyl moiety was replaced by other groups. In alkyl derivatives the unusual enhancement of the hydrolysis rate (exhibited by compound *36*) is maintained. However, a further increase of the side chain resulted in a reduction of both basicity and hydrolysis rate of the corresponding compounds, due probably to a hyperconjugation effect. Introducing a second methyl group in the *ortho* position in compound *37* (ie, 2,4-dimethyl-3-*N*,*N*-bis(2-chloroethyl)amino-benzoic acid, *44*) produced a further decrease of basicity without affecting the hydrolysis rate. It is likely that the first methyl group generated a rotation of the nitrogen mustard plane with respect to the aromatic nucleus plane, with an angle making imperceptible the effect of the second substituent.

From these compounds the 3-*N*,*N*-bis(2-chloroethyl)amino-4-methoxy-benzoic acid (27) (*41*, IOB-177, NSC-146172) was a potent inhibitor of a number of experimental neoplasms (table 6). Table 7 shows derivatives of compound *37* in which the chlorine atoms of the 2-chloroethyl moiety were replaced by Br and I. A decrease in the antitumor activity was noticed. When the *N*,*N*-bis(2-chloro-i-propyl)amino group was used instead of the 2-chloroethyl group as the alkylating moiety (compound *47*, IOB-340), a change of the ratio [k hydrolysis:k NBP alkylation]

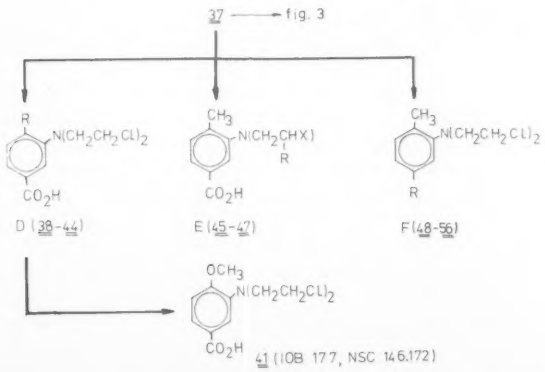


FIGURE 2

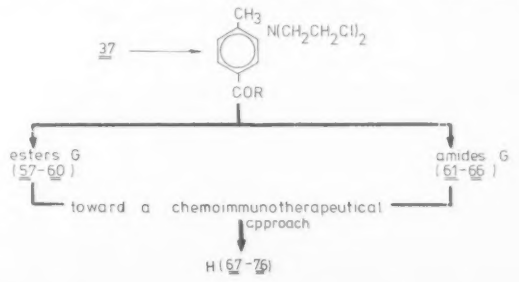
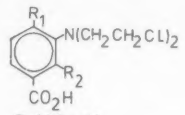


FIGURE 3

TABLE 5.—*o*-substituted aromatic nitrogen mustards derived from compound *37*



D (38–44)

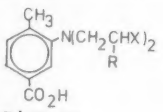
No.	R_1 =	R_2 =	Basicity of precursor amine (pka)	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antitumor activity (TGI%)	
						Jensen sarcoma	W256 carcino-sarcoma
38	C_2H_5	H	3.14 ± 0.03	3.50	0.130	95	59
39	n-C_3H_7	H	—	—	0.065	80	—
40	i-C_3H_7	H	3.31 ± 0.03	3.30	0.118	98	—
41	CH_3O	H	3.48 ± 0.02	3.05	0.056	96	60
42	Br	H	1.35 ± 0.05	3.63	0.146	96	—
43	Cl	H	1.56 ± 0.03	3.30	0.172	—	80
44	CH_3	CH_3	3.13 ± 0.03	3.32	0.052	98	—

TABLE 6.—Effectiveness of 3-*N*,*N*-bis(2-chloroethyl)amino-4-methoxy-benzoic acid (compound *41*) against experimental neoplasms*

Schedule (No. of doses)	Dose (mg/kg body weight)	No. of survivors/ No. treated	ILS			Cures
			T (days)	C (days)	T/C (%)	
		L1210/BDF$_1$				
3 doses × 4 days	18.7	5/6	10.4	9.3	111	
	12.5	6/6	14.5	9.3	155	
	8.30	6/6	13.0	9.3	139	
		P388/BDF$_1$				
9 daily	16.0	10/10	28.2	10.7	213	—
	8.00	10/10	16.4	10.7	153	—
	4.00	10/10	15.0	10.7	140	—
	2.00	10/10	15.8	10.7	147	—
		B16 melanoma/BDF$_1$				
9 daily	8.00	10/10	59.8	24.6	243	6
	4.00	10/10	39.0	24.6	158	—
	2.00	10/10	35.8	24.6	145	—
	1.00	10/10	30.0	24.6	121	—

*Screening was done by the National Cancer Institute.

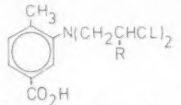
TABLE 7.—Side-chain modified derivatives of compound *37*



E (45–47)

No.	R =	X =	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antitumor activity (TGI%)	
					Jensen sarcoma	W256 carcino-sarcoma
45	H	Br	—	0.066	91	71
46	H	I	—	0.131	—	59
47	CH_3	Cl	2.71	0.112	—	68

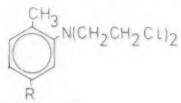
TABLE 8.—Comparative biologic and kinetic data for compounds *37* and *47*



37 R=H ; *47* R=CH_3

No.	Hydrolysis rate, log k66	NBP alkylation rate ($k80 \times 10^3$)	LD50 (mmols/kg body weight)	Antitumor activity: L1210 leukemia (ILS%)	Antitumor activity: W256 carcinosarcoma (TWI%)
37	3.33	110	0.063	50	86
47	2.71	97.5	0.112	84	68

TABLE 9.—Nitrogen mustards derived from compound *37*



F (*48*-*56*)

No.	R =	Basicity of precursor amine (pka)	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antitumor activity (TGI%): Jensen sarcoma	Antitumor activity (TGI%): W256 carcinosarcoma
48	SO_3H	—	—	0.640	53	0
49	$SO_2NC_4H_8O$*	2.62 ± 0.04	—	0.189	80	40
50	SO_2F	2.61 ± 0.04	3.96	0.320	—	45
51	PO_3H_2	2.90 ± 0.04	3.52	0.231	47	33
52	CHO	—	3.32	0.077	74	—
53	NO_2	2.32 ± 0.03	4.06	0.902	90	—
54	CO_2^- (Na+ salt)	—	—	0.091	—	56
55	CO_2^- $(NH_3CH_2C_6H_5)^+$	—	—	0.065	—	56
56	CO_2^- $(NH_3C_3H_7)^+$	—	—	0.087	—	88

* Morpholyl.

occurred. This may be rationalized in terms of lower electrophilicity of the resulting secondary carbocation, as in the case of the last compound (table 8). Table 9 shows derivatives of compound *37* in which the carboxylic group was replaced by other functions (F compounds, *48*–*56*). As expected, the introduction of more acidic groups completely ionized at physiologic pH greatly affects the antitumor activity (ie, SO_3H, PO_3H_2, etc). These facts were also in agreement with the properties ascribed to the carboxylic group.

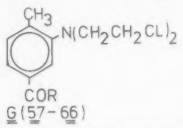
Amides and esters that were derived from compound *37* were synthesized and studied (G compounds, *57*–*66*) (table 10, fig 3). The high antitumor activity of some esters supported the possible in vivo hydrolysis of this function, previously observed in simple *meta*-substituted nitrogen mustards (*9*–*11*). This fact suggested the possibility of coupling the 3-*N*,*N*-bis(2-chloroethyl)amino-4-methyl-benzoyl moiety, or even the weaker reactive 3-*N*,*N*-bis(2-chloroethyl)amino-benzoyl moiety, with more selective carriers (steroids, chromanones, coumarins, etc), by way of an esteric bond in order to improve their selectivity toward malignant cells.

Some amides also exhibited marked antitumor properties, but generally they were less active than their esteric homologs.

Aromatic Nitrogen Mustards Capable of Coupling With Proteins

The selective toxicity of the antineoplastic agents toward malignant cells could be enhanced on the basis of a chemoimmunotherapeutic approach in two ways:

TABLE 10.—Esters and amides derived from compound *37*

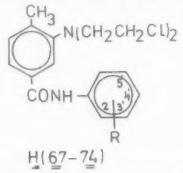


G (57–66)

No.	R =	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antitumor activity (TGI%) Jensen sarcoma	Antitumor activity (TGI%) W256 carcino-sarcoma
57	OCH_3	3.39	0.068	97	84
58	$OCH(CH_3)_2$	3.38	0.063	97	—
59	$OC_{17}H_{35}$	—	0.580	28	—
60	$O(CH_2)_2N(C_2H_5)_2$	—	0.053	90	67
61	NH_2	3.13	0.051	86	72
62	NHC_2H_5	3.19	0.038	96	87
63	NC_2H_4*	3.03	0.055	95	80
64	NC_5H_{10}†	3.02	0.146	93	36
65	NC_4H_8O‡	—	0.048	83	—
66	NHC_3H_2NS§	3.39	0.130	82	—

*Aziridinyl.
†Piperazinyl.
‡Morpholyl.
§Thiazolyl.

TABLE 11.—Aromatic nitrogen mustards capable of coupling with proteins



H (67–74)

No.	R =	Hydrolysis rate, log k66	LD50 (mmols/kg body weight)	Antineoplastic activity W256 carcino-sarcoma (TGI%)	Antineoplastic activity L1210 leukemia (ILS%)
67	2′-SO_2F	3.59	1.154	90	0
68	3′-SO_2F	3.54	0.230	45	15
69	4′-SO_2F	3.95	0.220	60	10
70	2′,5′ $(CH_3)SO_2F$	3.50	1.118	10	—
71	2′$COCH_3$	3.51	1.221	80	21
72	3′$COCH_3$	3.14	1.018	90	0
73	4′$COCH_3$	3.20	0.420	37	0
74	2′$COCH_2Br$	—	0.052	40	0
75	3′$COCH_2Br$	—	0.106	80	0
76	4′$COCH_2Br$	—	0.165	80	0

(1) By modifying the cell's surface. The structural features of such haptens were discussed by Soloway et al (28).

(2) By using antibodies as specific tumor-localizing agents. The enhancement of the antineoplastic activity of some cytostatics when admixed with immunoglobulins has been demonstrated (28,29).

To determine whether the immunoglobulins can transport selectively the cytostatic to the neoplastic cells, it will be necessary to conjugate the drug by a covalent linkage. Such attempts using alkylating agents and antimetabolites were reported, the conjugation usually being done by way of carboxyl and/or amino groups (30–32).

In order to increase the effectiveness of our compounds, the synthesis of new aromatic nitrogen mustards (H compounds, ***67–76***), capable of coupling with selected proteins while leaving the cytotoxic

moiety intact, was undertaken. Some of the results obtained are given in table 11 (11).

Transport Properties of the 4-Methyl-3-amino-benzoic Acid Molecule

It is very difficult to find good criteria to evaluate the carrier properties of a given structure. Our limited attempts to use the 4-methyl-3-amino-benzoic acid molecule for the transport of other cytotoxic groups failed (ie, compounds ***75*** [33] and ***78***,[7] both being inactive against L1210, 77%–66% TGI against W256 carcinosarcoma) (fig 4).

Supposing that the carrier specificity is related at least for a given range of chemical reactivity to an optimum log P, the substitution of the *N,N*-bis(2-chloroethyl)amino moiety with another cytotoxic group (table 12) can modify the transport characteristics of the entire molecule. This could be one of the reasons for which such compounds had only borderline effectiveness against the experimental neoplasms tested (34).

Mention should also be made that experimental difficulties appeared in the log P determination of nitrogen mustards in the water:octanol system (ie, hydrolysis of the parent compound occurring during the determination) (table 13).

Structure-Activity Correlations

Factors determining the selectivity of alkylating agents were recently discussed in a review by Ross (4). However, the following areas must be emphasized:

(1) Alkylation mechanism (ie, if true carbocations are involved or if a direct reaction with the nucleophilic center occurs). The alkylation of such centers is an anchimeric-assisted reaction in the case of nitrogen mustards and other alkylating agents. Therefore, it is possible to assume that the steric features and the reactivity of the carbocationic intermediates could have some influence on the biologic properties of such compounds. Some examples (4,7) and also our data support this point of view. Considerable attention must be focused on compounds able to generate more stable carbocations.

(2) The steric features of the reaction center. NBP alkylation represents only one possible pattern for such reaction describing the behavior of *N* centers in free purines and pyrimidines. Some mechanistic differences could occur when sulhydryl or phosphoric groups are involved, depending on steric characteristics of both nucleophilic centers and reacting intermediates. The same is true when nucleic acids are involved. However, the alkylation rate

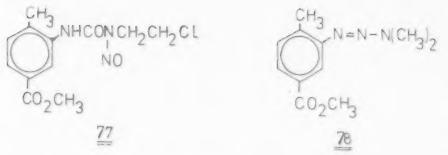


FIGURE 4

(determined by the NBP method) has until now been considered the best parameter for quantitative structure-activity correlations in this area.

The contribution of log P in describing the in vivo distribution of such compounds is clearly limited by their chemical reactivity. In this paper we use the hydrolysis rate as a correlation parameter, assuming that all studied nitrogen mustards exhibited the same alkylation mechanism. Relationships were found which reflect a direct dependence between toxicity and hydrolysis rate:

$$\log LD50 = 0.29\ (\pm\ 0.03) - 0.54\ (\pm\ 0.07) \log k66$$
$$(n = 10;\ r = 0.991;\ s = 0.034)$$
$$\log LD50 = 0.26\ (\pm\ 0.05) - 0.46\ (\pm\ 0.07) \log k66$$
$$(n = 21;\ r = 0.947;\ s = 0.105)$$

Part of the *m*-substituted derivatives B belong to the first group and part of the compounds of groups C, D, F, and G belong to the second group.

Compounds H (***67–76***) did not fit with this correlation; their LD50 could not be satisfactorily evaluated because of their poor solubility. They were administered as a suspension and generally the apparent toxicity was lower than the true one.

As expected, compound ***47***, alkylating through a secondary carbocation, failed to fit with such a correlation. No significant correlation was found between antitumor activity and the structural parameters investigated.

Our data suggested that the reactivity of the *m*-substituted aromatic nitrogen mustards could be to some extent correlated with their toxicity. The antitumor effectiveness against a given system is related in a much more complex way to σ, π, and other parameters (steric features and reactivity of ionic intermediates, transport factors, density of target sites, etc) which could not be satisfactorily evaluated.

TABLE 12.—Calculated π values of some alkylating moieties

No.	Alkylating moiety	Calculated π value
1	$N(CH_2CH_2Cl)_2$	1.3
2	$NHCON(NO)CH_2CH_2Cl$	0.2–0.3
3	$N = N - N(CH_3)_2$	0.5
4	$N = N - N(CH_2CH_2Cl)_2$	1.6
5	$N(CH_2CHClCH_3)_2$	2.3
6	$CON(CH_2CH_2Cl)_2$	0.9
7	$OCON(CH_2CH_2Cl)_2$	0.6

[7] Ionesco D, and Niculescu-Duvăz I. Personal communication.

TABLE 13.—Log P values for nitrogen mustards *37* and *47*

No.	Log P	
	Calculated*	Found†
37	3.57	3.38
47	4.57	4.28

*Starting from benzoic acid.

†The experimental values of log P were obtained from the data found in the octanol:water system taking into account the hydrolysis rate of the parent nitrogen mustards.

Recently, a QSAR analysis of a set of *p*-substituted aniline nitrogen mustards was published (35) and led to somewhat similar conclusions especially regarding the influence of ortho effects on both efficacy and toxicity of such compounds.

In contrast with our report, good correlations were also found between the antitumor activity and hydrolysis rate. These discrepancies might be assigned to the lack of suitable ED90 values for *m*-substituted nitrogen mustards, the inhibition at optimum doses usually being determined in our laboratory.

In order to make possible an accurate comparison with the before-mentioned data (35), a more refined mathematic analysis (involving supplementary parameters) of our set of *m*-substituted aromatic nitrogen mustards is in progress.

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Perturbed Cell Kinetics of 9L Rat Brain Tumor Cells Following Dianhydrogalactitol [1,2,3]

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SUMMARY

Kinetic changes induced by dianhydrogalactitol (DAG) in cultured 9L rat brain tumor cells were studied using conventional autoradiography and flow cytometry. Cell cycle parameters for the untreated cell line were: G_1, 8.5 hours; S, 8.2 hours; G_2, 3.2 hours; M, 0.5 hour; and cell cycle time, 19.5–20 hours. Treatment with 5 μg/ml of DAG, which caused 89.8% cell kill as measured by the colony-forming efficiency assay, resulted in the following effects: (*a*) undisturbed progression of G_2 cells toward mitosis; (*b*) disturbed progression of the cells in the S–G_2 boundary; (*c*) extreme prolongation of DNA synthesis time; and (*d*) temporary accumulation in G_2 phase of cells that were in G_1 or S phase at the time of treatment.

[Cancer Treat Rep 62:2055–2061, 1978]

A new hexitol epoxide compound, dianhydrogalactitol (DAG), has been reported to be a cell-cycle nonspecific agent which rapidly enters the brain and tumor intracellular compartments (1,2). DAG has been investigated in animal brain tumor systems (2) and in phase I tests in patients (3).

Interestingly, experimental brain tumors exhibit variable responses to this drug. It is most active against an ic inoculated ependymoma, less active against ic 26 glioma, and least active against ic rat 9L brain tumor (gliosarcoma) (2). These observations prompted us to investigate DAG-induced cell perturbations and to accumulate data that could be used to enhance the efficacy of DAG through more rationally designed drug regimens, as well as to aid in the scheduling of combination chemotherapy and/or radiation therapy. In the study described here, autoradiography and flow cytometry were used to analyze the effects of DAG on 9L rat brain tumor cells in vitro.

MATERIALS AND METHODS

The culture techniques used in this study were the same as previously described (4). The cell cycle parameters for the untreated cell line, as determined from the percent labeled mitoses (PLM) curve following a pulse of ^{3}H-thymidine (^{3}H-TdR), were: G_1, 8.5 hours; S, 8.2 hours; G_2, 3.2 hours; M, 0.5 hour; and cell cycle time, 19.5–20 hours. Unless otherwise stated, the following protocol was used to treat 9L cells in culture. Cells (1×10^6) were seeded into 75-cm^2 Falcon tissue culture flasks (Falcon Plastics, Oxnard, Calif) containing 10.0 ml of medium (90% Eagle's basal medium supplemented with 10% fetal calf serum). Cells were allowed to remain undisturbed for 48 hours to provide an asynchronous, exponentially growing culture for treatment with DAG. An automatic cell counter (Royco Instruments, Inc, Menlo Park, Calif) was used to count the

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[3]Tumor cells used in this study were provided by William H. Sweet, Paul T. Kornblith, Janette L. Messer, and Beverly O. Whitman of the Massachusetts General Hospital, Boston.

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cells. DAG was dissolved first in saline and then diluted in Hanks' calcium-magnesium-free balanced salt solution (BSS; GIBCO, Grand Island, NY) to make the stock solution. One ml of this stock DAG solution was added to treatment flasks to provide the desired concentration of DAG.

Dose-Response Study

Dose-survival responses were determined by the colony-forming efficiency (CFE) assay as described by Wheeler et al (4). Cells (1×10^6) were inoculated into a series of 75-cm^2 flasks and incubated for 2 days. Different doses (1, 2.5, 5, 7.5, and 10 μg/ml) of DAG were added to each culture bottle. After a 2-hour exposure to the drug, the DAG-containing medium was decanted and the cells were washed and trypsinized. Single cell suspensions were plated in 60-mm plastic Petri dishes (Microbiological Associates, Albany, Calif) containing 10^4 heavily irradiated (4000 rads) 9L feeder cells. After incubation for 14 days, the medium was removed and the colonies were fixed with absolute methanol. They were then stained with 2% Giemsa and counted. CFE was computed as 100 times the cloning efficiency of treated cells divided by the cloning efficiency of control cells. Colonies containing < 50 cells were not counted.

Effect of DAG on Growth Rate

Cultures containing 1×10^6 cells initially were treated according to the following schedule: (*a*) medium alone; (*b*) medium containing 1 μg/ml of DAG; (*c*) medium containing 5 μg/ml of DAG; and (*d*) medium containing 10 μg/ml of DAG. One milliliter of each stock solution of DAG was placed in treatment flasks containing tumor cells growing in 10 ml of medium. After treatment with DAG for 2 hours, cultures were washed twice with BSS (room temperature) and then allowed to grow in prewarmed (37°C) fresh medium. On 4 successive days after DAG treatment, three flasks from each experimental group were taken out and cells in each flask were trypsinized (0.25% trypsin [GIBCO] + 20 mg/100 ml EDTA [University of California Pharmacy]) at 37°C for 5 minutes and counted.

Autoradiography

Pulse chase method.—One and one-half hours after receiving 5 μg/ml of DAG, 0.1 microcurie/ml of ^{3}H-TdR (specific activity, 0.36 curie/mmol; Schwartz Bio-Research, Inc, Orangeburg, NY) was added to the cells for pulse labeling during the last 30 minutes of drug treatment. The cultures were then washed twice with BSS and allowed to grow in normal medium. The cells in three replicate flasks were harvested and fixed at 1.5–3-hour intervals. After fixation the cells were spread on microscope slides, air-dried, dipped in Kodak NTB-2 nuclear emulsion (Kodak, Rochester, NY), and exposed at 4°C for 10–14 days (5). After development of the coated slides, 50–100 mitoses stained with hematoxylin were examined, and a PLM curve was constructed. The mitotic index (MI) and labeled population (LP) were determined by scoring 1000–3000 cells from replicate slides. Because background grains were < 1–2 grains/nucleus, a nucleus containing > 4 grains was arbitrarily scored as labeled.

Double-labeling method.—Cultures were treated with 5 μg/ml of DAG for 2 hours, washed twice with BSS, and then allowed to grow in normal medium. Thereafter, at selected times (0, 2, 5, 8, 13, 23, 35, and 47 hours), 0.1 microcurie of ^{3}H-TdR (specific activity, 0.36 curie/mmol) was administered to three replicate flasks (as above) at each time interval, and, 2 hours later, 0.5 microcurie of ^{14}C-TdR (specific activity, 0.03 curie/mmol; Schwartz Bio-Research, Inc) was added. After a 15-minute incubation with both isotopes, cells from each of three flasks were washed twice with BSS and fixed separately. Autoradiographs were obtained as previously described (5). ^{14}C-labeled cells and cells labeled with ^{3}H only were scored by examining > 600 labeled cells from each slide. The labeling index (LI) (based on ^{14}C-labeled cells) and the rate of cell flux from S to G_2 (based on the percent of ^{3}H-labeled cells appearing per hour), as well as the relative clearing rate (RCR, compared to controls) of late S phase, were estimated using equation [1]:

$$\text{RCR} = \left(\frac{^3\text{H-labeled}}{^{14}\text{C-labeled}}\right) \Big/ \left(\frac{^3\text{H-labeled}}{^{14}\text{C-labeled}}\right)_{\text{control}} \qquad [1]$$

The reciprocal of RCR (1/RCR) gives the approximate duration of the flux period relative to the untreated control value of 1.0 (T_{FR} = relative flux period).

Flow cytometry (FCM) analysis.—One random sample from treated cultures prepared for the PLM study was removed from the incubator every 3 hours and the cells were trypsinized and fixed in 10% buffered formalin. The nuclear DNA was hydrolyzed with 4 N HCl at ambient temperature for 20 minutes before the cells were stained with acriflavine (0.02 g of acriflavine + 0.5 g of $K_2S_2O_5$ in 10 ml of 0.5 N HCl and 90 ml of distilled water) for another 20 minutes as previously described (6). These specimens were analyzed on the flow cytometer at the Lawrence-Livermore Laboratories of the University of California (7).

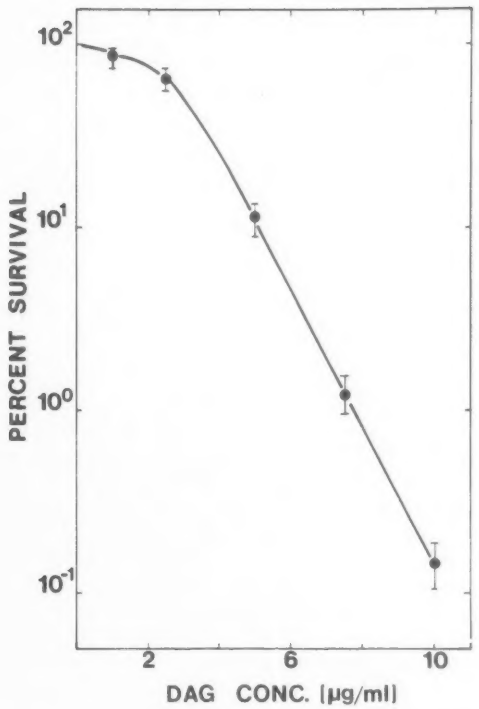

FIGURE 1.—Dose-response curve of 9L cells treated with DAG for a 2-hr exposure.

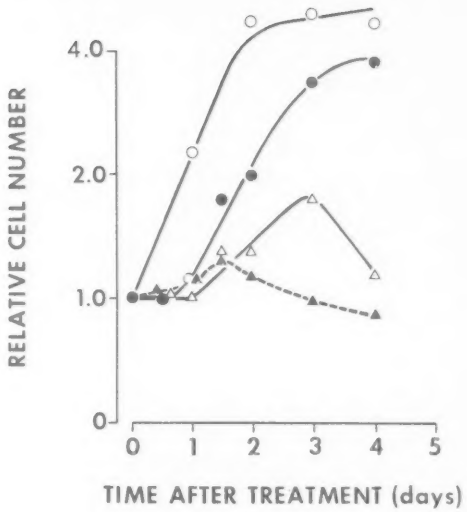

FIGURE 2.—Comparative growth curve of 9L cells in vitro. Drugs were added 48 hrs after cells were plated in Falcon plastic flasks. ○ = medium alone; ● = medium containing 1 μg/ml of DAG; △ = medium containing 5 μg/ml of DAG; ▲ = medium containing 10 μg/ml of DAG. Each point indicates the relative cell number calculated from flask.

RESULTS

Dose-Survival Response

The cloning efficiency of the untreated control cultures was 79.7% ± 4.2%. Survival of cells after a 2-hour exposure to DAG was 68.4% ± 6.2%, 8.9% ± 1.6%, and 0.12% ± 0.04% for 1-, 5-, and 10-μg/ml concentrations of DAG, respectively. The CFEs were calculated from these data, and the dose-response curve is shown in figure 1.

Growth Rate

The comparative growth curves of 9L cells with and without DAG treatment are shown in figure 2. Untreated cultures grew exponentially to approximately 6.5×10^6 cells/flask, after which the rate of growth decreased. Cultures treated with 1 μg/ml of DAG displayed growth inhibition during the first day of treatment; however, they proliferated at about the same rate as control cultures thereafter. DAG concentrations of 5 and 10 μg/ml caused significant inhibition of cell growth. This was manifested as progressive depletion by the third day of the cell population that had been treated with 5 μg/ml of DAG, after an initial population increase on the second day. It is noteworthy that in cultures treated with 5 μg/ml of DAG, the cells had almost doubled by the third day. After an initial 2-hour treatment at 1, 5, or 10 μg/ml of DAG, 90%–95% of the cells were capable of excluding trypan blue for periods up to 48 hours, indicating that they were still viable.

Autoradiography

Analysis by pulse chase method.—The PLM and MI curves are presented in figure 3. An appreciable increase in labeled mitoses was noted 3.5 hours after a 2-hour treatment with DAG (fig 3B), and may correspond to the duration of the G_2 phase. The PLM attained was nearly 100%, 7 hours after treatment with DAG, and then decreased gradually to 30%. There was no second wave of labeled mitoses observed during the entire 44.5 hours (fig 3B). It is not possible to estimate the duration of S phase from such a perturbed PLM curve; however, it was calculated by measuring the interval between the ascending arm and the descending arm of the PLM curve at the 50% level to be 21.7 hours. Analysis of the MI (fig 3C) revealed a gradual decrease to 50% of the control value by 10 hours;

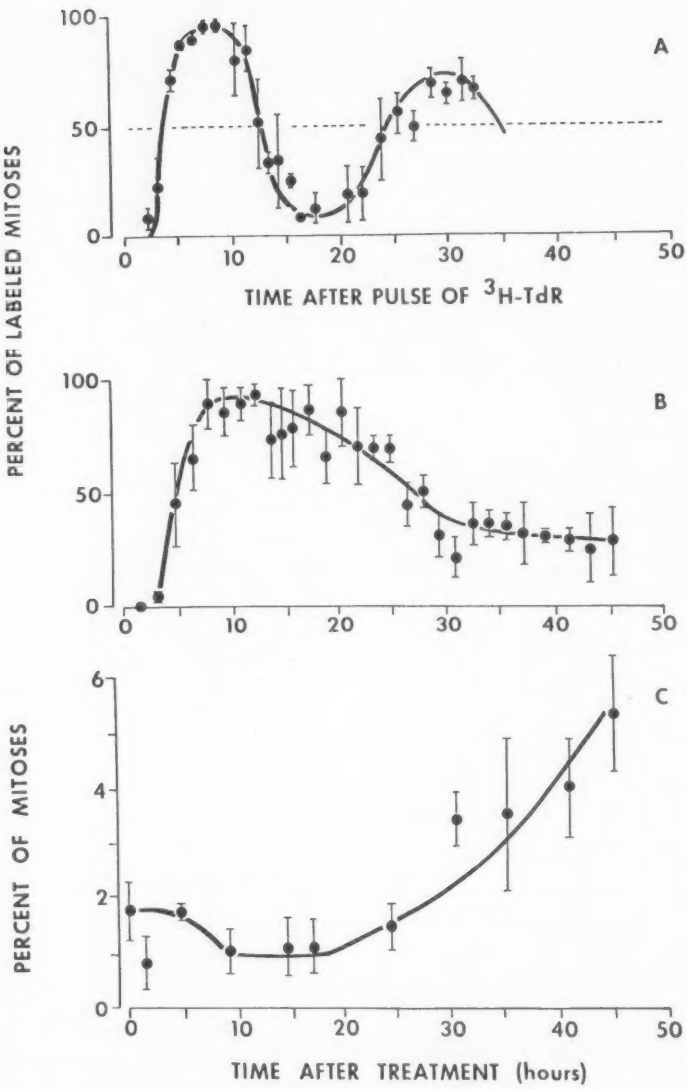

FIGURE 3.—(*A*) PLM of unperturbed 9L cells, (*B*) PLM of DAG-treated cells, and (*C*) % MI in drug-treated cells. Bars at each point represent SDs.

TABLE 1.—DAG-induced changes in LI and S phase transit time measured by double autoradiography*

Time after treatment (hrs)	LI by ^{14}C-TdR ± SD (%)	S–G_2 flux ± SD (%/hr)	RCR at S–G_2 boundary†	
			RCR	1/RCR
0	44.0 ± 1.7	5.4 ± 0.4	1.0	1.0
2	50.2 ± 4.5	3.8 ± 0.3	0.58	1.72
5	64.1 ± 3.0	3.4 ± 0.4	0.43	2.36
8	64.2 ± 4.6	2.9 ± 0.5	0.37	2.70
13	64.9 ± 0.91	4.5 ± 0.3	0.56	1.76
23	25.8 ± 7.2	3.3 ± 0.7	1.03	0.97
35	17.6 ± 2.8	1.3 ± 0.2	0.62	1.61
47	23.4 ± 2.2	1.3 ± 0.2	0.43	2.36

* After exposure to 5 μg/ml of DAG for 2 hrs.
†*See* Materials and Methods section for details.

the MI remained at this level for 8 more hours. From 18 to 44 hours, the MI increased to 6%, which was almost three times that of control cells. The LP of 42% ± 2.0% at 0 time increased to 52% ± 2.0% at 29 hours, before decreasing to the initial level of 42% ± 3.7% 40 hours after treatment (fig 4).

Analysis by double-labeling method.—The LI for ^{14}C-TdR increased from 44.0% at 0 time to 64% at 5 hours and remained at that level for 8 hours (table 1). From 13 to 23 hours the LI decreased to about 25% and stayed at this level for the next 24 hours. In contrast, cells effluxing from S to G_2 gradually decreased to 1.3% (of the total population)/hour with a temporary recovery around 13 hours after treatment. The RCR of late S phase cells into G_2 was reduced to about 50% of the control rate, with the exception of cells measured 23 hours after treatment.

FCM Analysis

Representative FCM profiles of DAG-treated 9L cells are shown in figure 5. The control profile (0 hour) exhibits a large peak on the left which represents cells with a 2C (G_1) DNA content. The smaller peak at the right represents cells with a 4C DNA content, an indication that the cells are in G_2 or M phase. The interval between the two peaks indicates that the cells in S phase have replicated varying amounts of DNA during their progression through this phase. Cells located near the 4C DNA peak

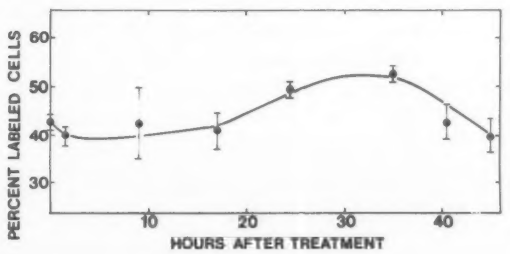

FIGURE 4.—LPs from the same samples used to calculate figs 3B and C. LP is the fraction of cells labeled at various times after a 30-min exposure to ^{3}H-TdR. Bars at each point represent SDs.

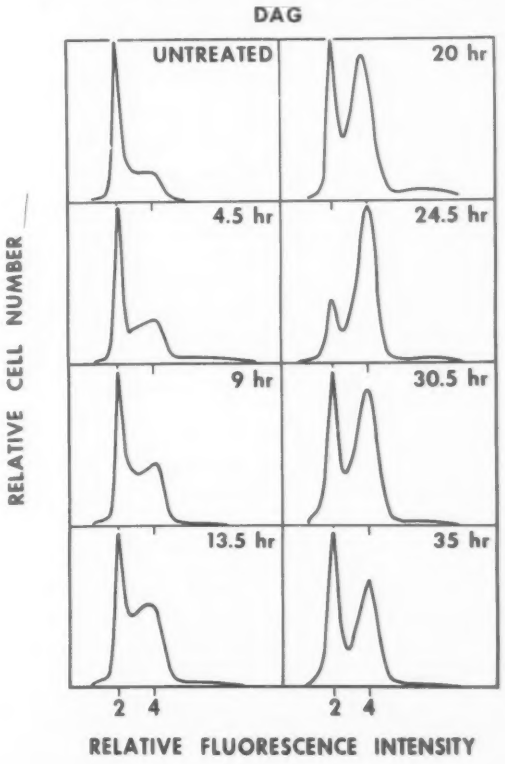

FIGURE 5.—FCM analysis of 9L cells treated with 5 μg/ml of DAG. Relative fluorescence intensity values of 1.0 and 2.0 correspond to the positions of nuclei with 2C and 4C DNA contents, respectively.

have replicated nearly a full complement of DNA and, therefore, are in late S phase.

DAG treatment resulted in a time-dependent alteration of this profile. The inversion of the height of the 2C and 4C peaks after treatment indicates that there was a significant increase in the number of G_2 phase cells during the first 24.5 hours. Thereafter, the second peak decreased gradually, in comparison with the first peak. Inhibition of DNA synthesis as well as blocking at G_2 were most obvious 24–30 hours after drug treatment. At 24.5 hours, the percentages of the population in G_1 and G_2–M phases were approximately 20% and 80%, respectively.

DISCUSSION

Asynchronous 9L rat brain tumor cells treated with DAG for 2 hours exhibited a shoulder threshold-type survival curve (fig 1), indicating that some damage may accrue before lethality is expressed. Figure 2 shows the comparative growth curves of 9L cells treated with 1, 5, and 10 μg/ml of DAG, respectively, for 2 hours. At a DAG concentration of 5 μg/ml, there was severe inhibition of growth, with only a minor increase in cell number that continued up to the third day; at this time the population had nearly doubled. At 10 μg/ml of DAG, growth was even more inhibited; after 48 hours there was no further increase in cell number, and there was actually a gradual decrease in cell number thereafter. Depletion of the cell population after this slight increase subsequent to DAG treatment may indicate that the majority of cells died after one cell division. The survival study of cells treated with 5 μg/ml of DAG for 2 hours revealed 89.8% cell kill by the CFE assay. We studied the perturbation of the 9L rat brain tumor at this schedule since prominent changes in cell kinetics were observed.

Analysis of the PLM curve does not indicate a direct effect of DAG on cells in G_2 since the time of appearance of labeled mitoses coincided with that in the control culture. In addition, there was no change in the MI during the first 5 hours. A dip at 1.5 hours was also seen in control cultures, suggesting that mitotic cells were lost during the washing procedure. The rate of accumulation of labeled mitoses was, however, found to be slightly retarded. This indicates that cells in late S phase at the time of exposure to DAG were temporarily delayed in their progression to G_2 phase, compared to untreated cells. An alternate interpretation is that cells were delayed in their progression from G_2 phase to metaphase, as observed by Barranco and Flournoy (8). However, we prefer the former interpretation, since the double-label autoradiography study revealed a definite delay in progression at the S–G_2 boundary. When cells were incubated in 5 μg/ml of DAG, stagnation of cells in the S phase was obvious, as demonstrated by the exaggerated first wave of the PLM curve, although the standard measurement of the S phase from the curve could not be made. Since the initial MI of 2% decreased to 1% during the first 20–25 hours after treatment, it appears that inhibition of DNA synthesis occurred mainly in those cells exposed to DAG during mid-S phase. An exact estimation of S phase duration could not be made from results of the double-labeling study because on-going changes in the number of cells influxing and effluxing from DNA synthesis may modify the length of S phase in an unpredictable manner. Nevertheless, this experiment revealed (*a*) a decreased number of cells coming out from S phase, while (*b*) late G_1 cells continued to initiate DNA synthesis as shown by an increase in LI (^{14}C-TdR) from 44% to 64% during the first 5 hours, and (*c*) the cells in late S phase spend twice as much time in S phase, compared to the control cells, shortly before they complete DNA synthesis. Furthermore, the decreasing number of cells undergoing the S–G_2 transition strongly suggests that the inhibition of DNA synthesis is greater in cells that are in mid-S phase when exposed to drug. Otherwise the S–G_2 flux should increase, even though it would be retarded, reflecting the expected age distribution of such a growing population. These observations are similar to those made by Barranco and Flournoy using synchronized CHO cells (8). A transient recovery of S–G_2 flux and the RCR at around 13–23 hours after treatment could be the result of a decreased cell influx into S phase, as demonstrated by (*a*) the decrease in LI and (*b*) the reduction of the G_1 population as seen in the FCM profile, as well as the relative increase of effluxing cells against all the cells undergoing DNA synthesis during this time period.

The decreasing slope of the descending arm of the PLM curve may be ascribed to the temporary blocking effect on the cells at the G_1–S boundary or possibly on the cells in early S phase as described by Barranco and Flournoy (8). In the absence of a block, the descending arm of the curve should be as steep as that of the control. However, we cannot confirm this until the variability in S phase duration induced by the drug is eliminated. The decrease of PLM to 30% shows that the cells in G_1 entered mitosis, in addition to the cells in S phase, because it indicates that the nonlabeled G_1 cells diluted the observed labeled mitoses. This was also confirmed by the fact that the LP gradually rose from 42% to 52% in the first 29 hours due to multiplication of labeled cells. This increase was then diminished

beginning 35 hours after treatment due to division of unlabeled G_1 cells. Pályi (1) studied the responses of HeLa cells after a 15-minute exposure to 50 μg/ml of DAG, and he also noted changes in the cell cycle parameters. His observations extended over the relatively short period of 24 hours, within which time G_1 cells did not enter mitosis, since 100% labeled mitoses were observed between 10 and 24 hours and the MI decreased gradually during the 24 hours, approaching 0. Our data are in agreement with these results. However, our experiments show in addition that mitosis resumed 30 hours after treatment. As demonstrated by FCM analysis, by 30 hours most cells were arrested in the G_2 phase. Therefore, the absence of a second peak in the PLM curve in the presence of many mitoses could be explained by the ability of cells in G_1 and S at the time of treatment to complete mitosis after temporary accumulation in G_2. This release, however, would not be on a first-blocked, first-released basis, but rather on a random basis from all the G_2 pooled cells. This hypothesis is further supported by the observation that the survival of cells treated with 5 μg/ml of DAG was > 90% in 48 hours, and that the original number of cells had doubled by the third day after treatment. This transient G_2 block in the first cell cycle differs from a BCNU-induced G_2 block, during which most cells except those in early G_1 at the time of drug administration progressed through mitosis in the first cell cycle and accumulated at G_2 phase in the second cycle after treatment (9). Cell death at 5 μg/ml of DAG occurred at least one cell division after contact with DAG. Of importance from the chemotherapeutic standpoint is knowing in which phase the cells that survive such DAG treatment accumulate. Whether these cells are located in a specific phase of the cell cycle or are distributed throughout the cell cycle may affect the proposed additional treatment regimen.

The results of our study do not explain why DAG had little effect on the 9L brain tumor in vivo (2). The modest dose-response relationship of 9L cells and DAG, compared with other drugs such as BCNU and CCNU may be responsible. It is not known if this major difference in efficacy of the drugs towards 9L cells is the result of (*a*) a cell cycle perturbation that occurs only in vivo, (*b*) a drug delivery problem in rat brain, or (*c*) differing catabolic processes for the breakdown of DAG in vivo and in vitro.

Nevertheless, it is obvious that analyses of perturbation caused by drugs such as DAG in vitro can provide information which will aid in improving the design and scheduling of cancer treatment that involves the use of drug combinations and/or radiotherapy.

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Experimental and Clinical Studies on the Formation of Antibodies to Neocarzinostatin, a New Protein Antibiotic [1,2]

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SUMMARY

The injection of clinical doses of neocarzinostatin (NCS) in guinea pigs did not result in antibody formation as judged by immunoelectrosyneresis, micro-Ouchterlony agar diffusion, fluorescence polarization, and passive cutaneous anaphylaxis reaction. This confirmed previous work on passive hemagglutination and passive cutaneous anaphylaxis reaction in a heterocytotropic system in guinea pigs. Forty-eight serum samples from 28 patients who were previously treated with NCS alone (8–161 mg) for a period of 8–85 days did not show any sign of antibody formation as revealed by immunoelectrosyneresis, micro-Ouchterlony agar diffusion, and fluorescence polarization techniques. In a homocytotropic system, the passive cutaneous anaphylaxis reaction was carried out with sera of sensitized guinea pigs and test guinea pigs which revealed that no IgE and IgA antibody to NCS was present in the sensitized sera. Those patients with bladder cancer who did not respond to NCS therapy or exhibit any side effects even after 21 mg were found to have proteolytic activity in their sera which degraded NCS very rapidly as revealed by the fluorescence polarization technique.

[Cancer Treat Rep 62:2063–2070, 1978]

Neocarzinostatin (NCS) is an acidic single-chain polypeptide with a molecular weight of 10,700 composed of 109 L-amino acids (1). Clinically beneficial effects on gastric cancer, pancreatic cancer, leukemia, and bladder cancer have been reported (2–5). Because of its protein nature, the antigenicity of NCS, if any, could cause problems in clinical use. Furthermore, we have observed that a small percentage of patients receiving NCS showed a lack of antitumor activity and also were totally free from toxic effects (5). It seemed possible that this lack of activity might have resulted from the neutralization of NCS by antibodies. To clarify this point, we have used several techniques to study antibody formation in animal and patient sera. The experimental conditions were designed to reveal the role of the antigen-antibody reaction in the clinical use of NCS.

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MATERIALS AND METHODS

Sensitization with NCS and anaphylaxis in guinea pigs.—Seven to eight male Hartley guinea pigs per group, weighing 290 to 440 g each, were used. They were divided into four groups for different schedules of sensitization with NCS or ovalbumin followed by challenge with the antigen to elicit an anaphylactic reaction. Sensitization of the guinea pigs with a dose of 0.1 mg/kg (close to the clinical level) of NCS (Kayaku Antibiotic Research Laboratories, Tokyo, Japan) or 2.5 mg/kg of ovalbumin (E. Mark AG, Darmstadt, Germany) as a positive control was performed sc. Schedules of sensitization were as follows (table 1): *groups 1–3*—

TABLE 1.—Dose and schedule of antigen sensitization and challenge*

Group	Sensitization (sc)	Challenge (iv)	Methods of sensitization and challenge at wk— 1	2	3	4	5	6
1	NCS, 0.1 mg/kg	NCS, 0.5 mg/kg	S	S	S	S	S	
					C	C	C	
2	NCS, 0.1 mg/kg + adjuvant	NCS, 0.5 mg/kg	S	S	S			
						C	C	
3	NCS, 0.1 mg/kg + adjuvant	NCS, 0.5 mg/kg	S	S				
					C	C	C	
4	Ovalbumin, 2.5 mg/kg + adjuvant	Ovalbumin, 10 mg/kg	S	S				
					C	C	C	C
Blood collection (2 times/wk)								

*Adjuvant = Freund's complete adjuvant. S = sensitization with antigen once/day × 4; C = challenge with antigen once/wk.

NCS was given on 4 successive days/week for a period of 5 weeks (group 1), 3 weeks (group 2), and 2 weeks (group 3); *group 4*—ovalbumin was given on 4 successive days/week for 2 weeks. Freund's complete adjuvant (Difco Laboratories, Detroit, Mich) was used in groups 2–4. Two to 5 weeks after completion of immunization, excess antigen, 0.5 mg/kg of NCS, or 10 mg/kg of ovalbumin for the control group was injected once a week iv via the femoral vein and anaphylactic reactions were observed (table 1). During the experiments, serum samples for antibody assays were obtained by heart puncture two times per week and were stored in a deep freezer until assay.

Preparation of antibody against NCS for positive control.—Antibody (anti-NCS antibody) was a gift from Japan Immunoindustries Co (JIMCO, Takasaki, Japan) and was prepared against NCS in rabbits. Briefly, 3 mg of NCS and 3 mg of poly-L-lysine (Sigma Chemical Co, St. Louis, Mo; molecular weight, about 10,000) were dissolved in 0.01 M phosphate buffer of pH 6.0. Then, 1.6 mg of 1-ethyl-3-dimethylaminopropyl-carbodiimide HCl (Protein Research Foundation, Osaka, Japan) was added and allowed to react for about 3 hours under stirring at room temperature. After dialysis against phosphate buffered saline (PBS) (0.01 M phosphate buffered 0.15 M NaCl solution, pH 7.2) to remove reagents and to terminate the reaction, the polylysine-conjugated NCS was injected three times in the footpad of rabbits with Freund's complete adjuvant with an interval of 2 weeks between each injection.

Passive cutaneous anaphylaxis (PCA) reaction in a homocytotropic system.—The PCA reaction was carried out as previously described (6). Diluted or undiluted sera (0.1 ml) from sensitized guinea pigs were injected sc in guinea pigs, and excess antigen (NCS) was injected after 15 hours together with 1% Evans blue dissolved in 1 ml of saline by injection iv via the tail vein. After 20 minutes the animals were killed and the skin was opened. The permeability of the dye in the epidermal tissues was measured as a result of the presence of anti-NCS antibody, primarily IgE class. As a positive control, PCA was performed similarly using antigen (ovalbumin) and sera from ovalbumin-treated guinea pigs.

Micro-Ouchterlony (MO) double agar diffusion method.—The diffusion plate was prepared in the same way as described by Ouchterlony (7). Agarose gel (Nakarai Chemical Ltd, Kyoto, Japan) at 1% in 0.05 M tris-HCl buffer, pH 8.6, was used and six small circular wells (4 mm in diameter) were prepared circumferentially around a center well. In the center well, a small drop of solution with the antigen was placed and in the circumferential wells patient sera or sensitized sera were placed. After incubation for 20–48 hours at 4°C, precipitin lines were examined.

Immunoelectrosyneresis (IES).—IES was carried out according to the method described by Bussard and Huet with slight modification (8). The agar-agarose gel plate, which contained 0.6% of J. agar (Nakarai Chemical Ltd) and 0.6% of Agarose A–37 (Nakarai Chemical Ltd) in barbital buffer (0.05 M, pH 7.8), was prepared on a horizontal glass plate (2.5 × 7.5 cm^2). A pair of wells 4 mm in diameter, one for the antigen and the other for antisera, were punctured longitudinally. Each pair of wells was about 7 mm from the respective rims. Sera or antisera were placed on the cathode side. As a positive control, anti-NCS rabbit sera diluted 100-fold were used. After 45 minutes of IES at 0.8 milliampere/cm, 300 volts/cm, precipitins were examined.

Fluorescence polarization technique.—The fluorescence isothiocyanate (FITC) (Sigma Chemical Co)

labeling of NCS was carried out according to the method reported previously (9) in which only the α-NH_2 of the *N*-terminal alanine of NCS is labeled and the antigenicity remains. Fluorescence polarization (obtained as P value) was measured with JIMCO polarization spectrofluorophotometer Model MAC-2 HR-1. Details of the theoretic background (10,11) with instrumental information (12,13) have been described recently. Briefly, when the fluorochrome-labeled compound and confronting molecules interact (antigen-antibody interaction), the changes of molecular weight resulted in an increase in P value. The measurement was carried out in PBS at 20°C. The temperature was controlled with a refrigerated water circulator Model RTE-8 (Neslab Instruments Inc, Portsmouth, NH) within ± 0.01°C. FITC-labeled NCS (0.08 pmol in 50 μl) and serum (50 or 100 μl) were added to 2 ml of PBS. A standard curve was obtained by using anti-NCS antibody instead of serum. Serum samples from ten healthy persons were measured similarly to evaluate nonspecific changes of the P value such as viscosity (fig 1). When the P value obtained was abnormally high (> 2 SDs of normal control sera), a blocking with unlabeled antigen (NCS) was carried out before addition of labeled antigen to the system. If the P value increased even after the addition of unlabeled antigen, which will block the antigen-combining site of the antibody, then such a high value was interpreted as nonspecific binding or abnormally high serum viscosity, but was not felt to be due to antigen-antibody reaction.

Clinical studies.—Forty-eight venous blood samples were obtained from 28 patients who were treated with varying amounts (8–161 mg) of NCS. The schedules of NCS treatment varied. Protocols for NCS treatment were as follows: (*a*) daily iv injection of 1 mg of NCS as a single dose for a period of 7 days as one course in 17 patients (bladder cancer) (the total dose was 8–28 mg for 15–49 days); (*b*) weekly drip iv infusion (DIV) of 4 mg of NCS in three patients (gastric cancer) (the total dose was 32–36 mg for 50–57 days); (*c*) daily DIV of 2 mg of NCS in six patients (bladder cancer) (the total dose was 14–161 mg for 8–85 days); and (*d*) daily DIV of 4 mg of NCS for 4 successive days in two patients in 2 weeks as one course (pancreatic cancer and acute lymphatic leukemia) (the total dose was 40–48 mg for 30–39 days). Blood samples were collected at least 3 weeks after the treatment. Furthermore, in five patients serial sampling was carried out during and after treatment. Sera were separated after clotting by centrifugation at 4°C and held in a deep freezer (–30°C) until assay.

Evaluation of ineffective cases.—Most of the patients with bladder cancer treated with NCS showed good responses to the drug, but a few patients (about 10%) did not respond to the NCS treatment at all (5). To clarify this problem, the fluorescence polarization technique was used (13). When NCS is degraded by a proteolytic enzyme, the molecular weight of NCS becomes smaller. Therefore, when FITC-labeled NCS is incubated with sera which possess high proteolytic activity, the P value of FITC-labeled NCS decreases. Therefore, FITC-labeled NCS (0.08 pmol in 50 μl) and patient sera (50 μl) were mixed in 2 ml of PBS and the P value was measured. Sera from patients who were treated with NCS were collected after treatment and kept in a deep freezer until use.

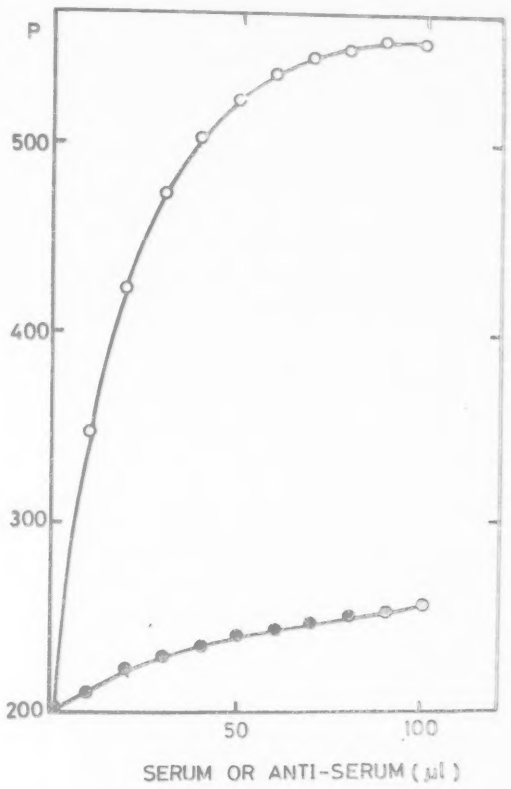

FIGURE 1.—Quantitation of antibody using the fluorescence polarization technique. Open circles = standard curve with FITC-labeled NCS (0.08 pmol as antigen in 2 ml of PBS) and varying amounts of established anti-NCS antibody (1.2 μg/μl as an antibody protein). Solid circles = control curve with increasing amount of normal sera from 10 healthy men and FITC-labeled NCS. Arbitrary P values obtained from normal sera (control) were 242.4 ± 4.3 (with 50 μl of serum in 2 ml of PBS) in contrast to 523.7 ± 6.2 (50 μl in 2 ml of PBS) with antibody-containing sera.

RESULTS

Anaphylactic reaction.—In groups 1–3, an anaphylactic reaction was not observed when challenge was carried out with excess antigen. Specifically, in group 1, challenge was carried out during sensitization. The anaphylactic reaction, however, was not observed symptomatically. The same results were obtained in groups 2 and 3 with challenge after completion of sensitization. In group 4, which was a positive control group with ovalbumin, anaphylactic reactions occurred after challenge on the third week. The results are arbitrarily expressed as 1+ to 4+ as judged by intensity of symptoms (*see* table 2). On the third week, symptoms such as palpitation, chills, and urinary incontinence were observed in two guinea pigs. Furthermore, four of seven guinea pigs died from anaphylactic shock. However, in group 4 after the fourth week, no anaphylactic symptoms were observed after challenge. In groups 1–3, all of the animals died after two challenges from the toxicity of the drug used for challenge. Especially in group 1, sensitization and challenge on the fifth week could not be carried out (table 2). In the clinical studies, anaphylactic reactions have not yet been seen in patients who received an average total dose of about 32 mg, although side effects such as anorexia, nausea, and slight weight loss were observed in most patients. Only one patient developed shock after a single iv injection of NCS, but it was not determined that NCS played a role in the antigen-antibody reaction since the patient was very emaciated.

Passive cutaneous anaphylaxis.—The size and intensity of the permeated dye (Evans blue) area were measured. No permeation of the dye in the skin was observed when serum samples from NCS-sensitized guinea pigs, with or without Freund's complete adjuvant, were injected (fig 2B). In contrast, severe permeability of the dye was observed in the ovalbumin-sensitized cases. Furthermore, intensity and area of the dye were correlated with dose of the sensitized sera. Diluted serum showed weaker spots (fig 2A). When tenfold diluted serum was injected, the average diameter was 17.2 mm, while 100- and 200-fold diluted serum resulted in an average diameter of 9.2 and 7.4 mm, respectively.

Immunoelectrosyneresis and micro-Ouchterlony.—By either of these methods, precipitin was not observed between NCS and sera from sensitized guinea pigs under various conditions and from patients treated with NCS. Nonspecific precipitin around the wells disappeared during the immersion in 0.85% saline for 4 hours. In contrast, clear precipitin was revealed between anti-NCS antibody (control serum) and the antigen (NCS) as well as between the sera from ovalbumin-treated guinea pigs and the antigen (ovalbumin) (fig 3).

Fluorescence polarization technique.—The limit of detection by this method was < 0.1 μg of IgG in 50 μl of serum. As shown in figure 4, most of the patients' sera were within normal limits (within 2 SDs of P value of control sera, solid circles). However, eight sera specimens from three individual patients showed high P values (open circles). Blocking with nonlabeled NCS was carried out with these

TABLE 2.—Anaphylaxis after challenge with antigen*

	Wk				
Group	2	3	4	5	6
1		C (–) 7/7	C (–) 7/7 (D) 7/7		
2			C (–) 7/7 (D) 2/7	C (–) 5/5 (D) 5/5	
3	(D) 1/8	C (–) 7/7 (D) 1/7	C (–) 6/6 (D) 6/6		
4		C (–) 1/7 (+) 2/7 (4+) 4/7	C (–) 3/3 (D) 1/3	C (–) 2/2	C (–) 2/2 (D) 2/2

* *See* table 1 for sensitization schedule. C = challenge with antigen once/wk; D = death caused by toxicity in 1 wk. Symptoms: – = no symptoms; + = symptoms of anxiety, chills, urinary incontinence, and palpitation; 2+ = symptoms of dyspnea and motor disturbance; 3+ = convulsion with other symptoms; 4+ = death caused by anaphylactic shock; (–) 1/7 and (4+) 4/7 = anaphylaxis was negative in 1 of 7 guinea pigs, and anaphylaxis was grade 4+ in 4 of 7 guinea pigs, respectively.

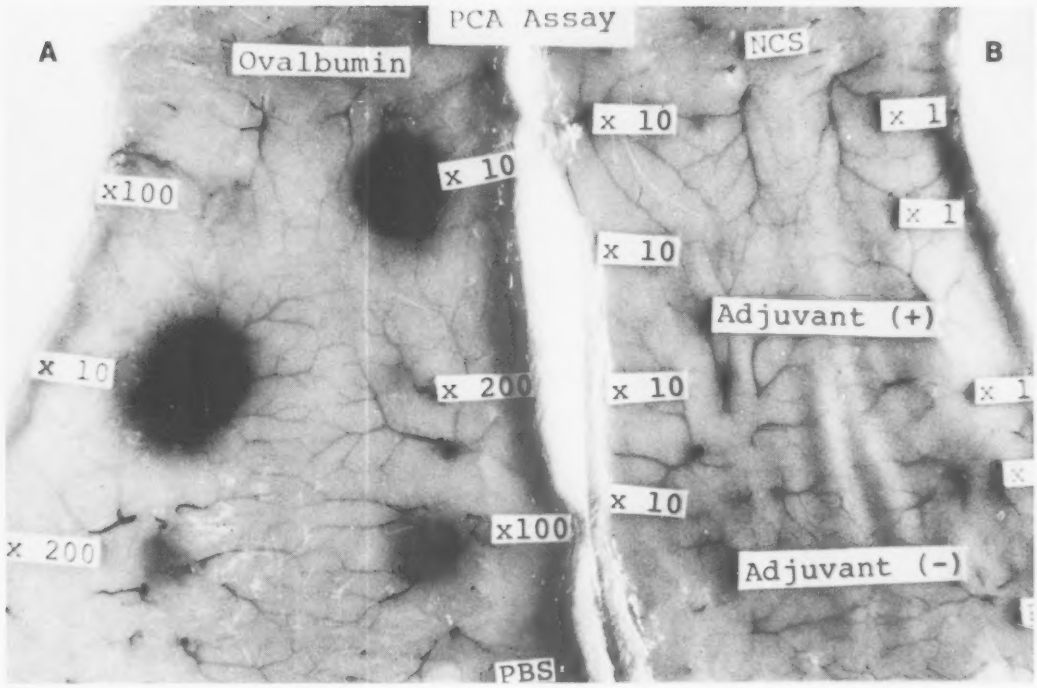

FIGURE 2.—PCA in homocytotropic system in guinea pigs. Permeated dye was observed clearly in the skin of control animals (ovalbumin and antiovalbumin) (A). In contrast, there was no permeated dye in the skin where NCS-sensitized sera had been injected and NCS was injected iv (B). x 10, x 100, and x 200 = dilutions of sera with saline.

sera, but none of these showed a decreased P value after blocking. This was interpreted to mean that the viscosity of the patient sera was elevated. In the case of sensitized guinea pig sera, there was no increase in P value to indicate NCS antibody formation. Therefore, it seems that antibody to NCS was not formed in either experimental animals or clinical patients or it was at an undetectable level. FITC-labeled ovalbumin lost its antigenicity, thus the fluorescence polarization method was inapplicable.

Evaluation of ineffective sera.—The changes in P value of FITC-labeled NCS were monitored as shown in figure 5. FITC-labeled NCS alone showed a constant P value during incubation for 1 hour at 20°C (arbitrary P value = 200, 0.08 pmol of FITC-NCS in 2 ml of PBS). When labeled NCS and sera from patients were mixed, P values were unchanged or changed due to nonspecific phenomena such as very high viscosity of the serum. Therefore, the initial P value was expressed as 100% in order to compare the reduction of P value after an incubation period. P values of labeled NCS were not decreased or were decreased only slightly during incubation for 35 minutes with sera from those patients who responded to NCS. Namely, the average P values in the effective cases decreased from 100% to 98% after 35 minutes. In contrast, P values decreased severely during incubation with sera from patients in whom NCS was ineffective even at a very low serum concentration. The average P value was 87% after 35 minutes. This indicates that such a decrease in P value reflects a decrease in the molecular weight of labeled NCS (13). Thus, the molecular weight of NCS after incubation was calculated using the diagram described by Maeda (13). Approximate molecular weight, after incubation with sera from effective cases, was 10,700 (unchanged) or > 9700, while that with sera from ineffective cases was 8200–6500 (about 69% of the initial molecular weight). This indicated a rapid degradation of NCS by the sera from nonresponding patients even at an extremely low serum concentration (fig 5).

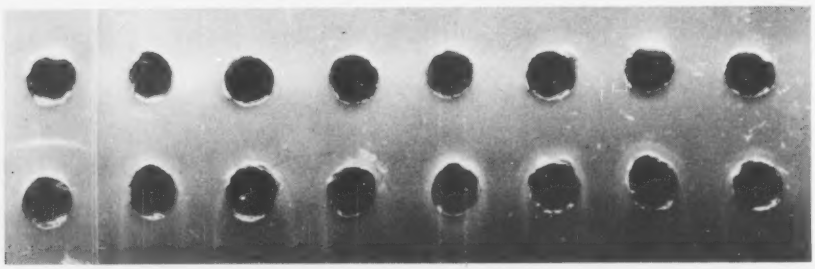

FIGURE 3.—IES (counter electroimmunophoresis). Upper wells (anode side) were filled with respective antigen (NCS: 1.5 μg/well), and lower wells were filled with sera from patients and control sera. After 45 mins, precipitin was observed in the positive control (extreme left), but there was no clear precipitin between antigen and patient sera (remaining 7 wells at right).

DISCUSSION

NCS is the first antibiotic of a protein nature to be applied clinically for cancer chemotherapy. In view of this, most clinicians feel a certain degree of insecurity about its potential antigenicity. NCS has been used for > 700 cases of gastric cancer alone in Japan, but no explicit case of an adverse effect due to an antigen-antibody reaction has yet been reported. Two or three cases with mild anaphylaxis-like symptoms associated with the administration of NCS were, however, observed. We have undertaken the present investigation in order to clarify the possible antigenic effect in guinea pigs and also in patients who were receiving NCS therapy.

Antibody formation to NCS was found to be negative when NCS with or without Freund's complete adjuvant was injected for 2–4-week periods in guinea pigs. NCS conjugated with poly-L-lysine, however, formed a high titer of antibody, which is detectable with MO, IES, or the fluorescence polarization technique even after 100-fold or more dilution. Sera from patients who had received an average of 32 mg (8–161 mg total), with a median term of NCS therapy of 28 days, showed no indication of antibody formation based on the results of MO, IES, or the fluorescence polarization technique.

Guinea pigs showed no anaphylaxis after sensitization with NCS under various schedules followed by challenge with excess antigen. Animals tolerated the dose levels used for the sensitization which is about the same or twice the dose levels used in therapy for bladder cancer and gastric cancer. However, the dose used for the challenge to induce anaphylaxis was so high that some animals showed toxicity after ≥ 2 weeks but not anaphylaxis, and all of the animals died during the experiment. The cause of death in NCS-sensitized guinea pigs was not a result of antibody formation (shock due to anaphylaxis as acute phenomenon) but a result of toxicity since the guinea pigs became feeble and weight loss was observed after sensitization and challenge which is typical death caused by the toxicity of NCS.

A PCA test was carried out using sera from

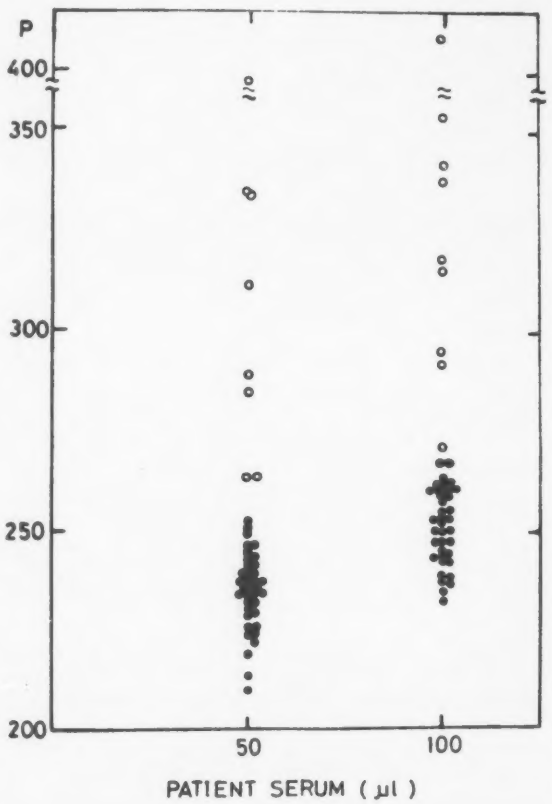

FIGURE 4.—Results of assay of antibody using fluorescence polarization technique. P values of most of the patient sera were within normal limits (within 2 SDs, solid circles), but a few showed exceptionally high P values (open circles).

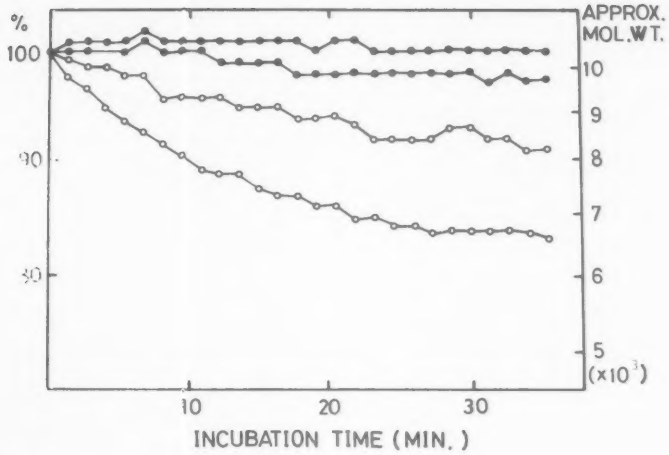

FIGURE 5.—Change in P value with sera from unresponsive patients. Effective patients who responded to NCS therapy showed no change or slight change in P value with their sera (solid circles), but with sera from patients who did not respond to NCS therapy there was a reduction in P value during incubation (open circles). Approximate molecular weight of degraded NCS is shown on the ordinate. Rapid degradation of FITC-labeled NCS was observed with the sera of those who failed to show chemotherapeutic effect of NCS due to proteolysis.

sensitized guinea pigs and healthy guinea pigs. PCA is not only one of the most sensitive assays for the detection of immunoglobulin, but also the schedule of antigen challenge, namely 18 hours after intradermal inoculation of the sera which may or may not contain the antibody, was designed to identify IgE and IgA antibody. This result again gave negative results for IgE class antibody formation to NCS.

All the above results indicate that NCS is extremely low in antigenicity or devoid of antigenic activity when given to guinea pigs or patients with or without Freund's complete adjuvant at a dose schedule close to that used clinically. Therefore, the present results may be explained by the following factors. First, NCS is excreted very rapidly into urine (50% excretion is achieved in 5 minutes) (14). Second, it is degraded quite rapidly by proteolytic enzymes in the blood (15) or in the liver (16). Third, it has a potent immunosuppressive activity which will hinder immunogenicity (17). Furthermore, patients receiving NCS were those with cancer and they are known to have impaired immunologic potency.

Toki reported similar results in 43 leukemic patients who had received total doses of NCS ranging from 8 to 174 mg for a period of 4–84 days (18), but he could not demonstrate any antibody titer as judged by the passive hemagglutination test or PCA in guinea pigs. In rabbits, when NCS was injected alone it failed to produce antibody, but with Freund's adjuvant Toki detected antibody titer dependent upon the schedule of NCS administration. Rabbit antibody to NCS was confirmed by PCA in the guinea pig as well, but its confirmation in guinea pigs using rabbit sera (antibody) may have a drawback because it was carried out in a heterocytotropic experiment.

Using the fluorescence polarization method we have observed that the apparent molecular weight of NCS, as revealed by P value, did not change with the sera from patients in whom NCS was effective (bladder cancer), but it was found to change with sera from patients in whom NCS was ineffective. Sera from two patients who did not respond to NCS therapy brought about degradation of NCS molecules even at a serum concentration of 2.5% (fig 5). It is, therefore, very interesting that degradation of NCS by sera appears closely related to the clinical effectiveness. These two patients showed not only no response to therapy but also no side effects at all. Therefore, use of urokinase or plasmin activator in the patients undergoing NCS therapy must be carefully controlled.

When a drug of a protein nature is administered, antibody to the drug is known to reduce therapeutic efficacy. Circulatory antibody was detectable in the case of L-asparaginase and also in the growth hormone (19,20). In our series, however, antibody to NCS was not detected in the experimental and clinical studies perhaps because of the reasons previously stated. With the fluorescence polarization method, three patients showed abnormally high P values, but they did not change upon blocking

with unlabeled NCS due to the extremely high viscosity of their sera, probably associated with fibrinolysis. One of them was undergoing urokinase therapy during his course of cancer treatment.

The above results show that NCS possesses no or very little antigenic activity as revealed by guinea pigs and patients using various methods.

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BCNU-5-Fluorouracil Combination Therapy for Recurrent Malignant Brain Tumors [1,2]

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SUMMARY

Twenty-nine patients with recurrent malignant brain tumors were evaluated. BCNU (180 mg/m^2) was administered iv on Day 1 and 5-fluorouracil (1 g/m^2/day) was given by continuous infusion on Days 15–17. Courses were repeated every 6 weeks until tumor progression occurred. Before each course, a neurologic examination and radionuclide and computerized tomographic scans were performed. Response and progression were defined as improvement or deterioration, respectively, in at least two of the three tests. Nine of 29 patients (31%) responded to therapy, five of 29 (17%) showed progression, and 15 of 29 (52%) had disease stability. The estimated median time to progression was 27 weeks for all patients, 34 weeks for the response group, and 25 weeks for the stable group. Of all 29 patients, 24 (83%) had tumor progression arrested; 40% with response and 6% with stable disease were continuing therapy after 1 year. The stable disease group was larger and the duration of stability was longer than that seen in previous studies. Evaluated by time to progression, a BCNU-5-fluorouracil combination is superior to either BCNU alone or a BCNU-procarbazine combination.

[Cancer Treat Rep 62:2071–2076, 1978]

We report here a nonrandomized phase II clinical trial of BCNU and 5-fluorouracil (5-FU) used in combination for the treatment of recurrent malignant gliomas. In 6-week courses, BCNU was given on Day 1 followed by a 72-hour infusion of 5-FU beginning on Day 15. Patients were treated until there was evidence of tumor progression.

The study was based on evidence that a cell-cycle phase-specific (CCS) drug given during active tumor cell repopulation would not only reduce the tumor cell population, but would also prevent tumor proliferation for 4–5 days.

BCNU is a proven antitumor agent that alkylates nucleic acids and is cell-cycle nonspecific in its mode of action. In a previous clinical study of BCNU for recurrent malignant glioma, 17 of 37 (47%) patients responded to therapy or had stable disease for a median duration of 9 months (1,2). Clinical and experimental studies have shown the value of BCNU as one component of combination therapy protocols.

Rosenblum et al found that a single ip injection of BCNU at 75%–100% of the LD10 dose produced a 3–4-log cell kill in intracerebral (ic) 9L gliosarcoma tumors in rats (3,4). Under the conditions of Rosenblum's experiments, no significant difference was noted between single injections and closely spaced fractionated injections (4). Tumor repopulation began about 2–4 days after a single injection of BCNU and was exponential for the next 4–12 days, suggesting that administration of an appropriate CCS drug

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during exponential tumor cell repopulation might maximize the cell kill of S-phase cells.

The pyrimidine analog, 5-FU, used extensively for the treatment of colorectal cancers (5), was chosen as the CCS drug. 5-FU crosses the blood-brain barrier (6,7) but is not active against ic tumors in animals (8,9) or brain tumors in man (10) because of the low growth fractions of these tumors (11,12). Thus, if tumor cells were exposed to the drug for the entire generation time, neither a 1-log cell kill nor a significant increase in survival time could be expected (3).

Based on the results of Rosenblum et al (3,4), 5-FU was given to ic glioma 26 and 9L tumor-bearing animals after administration of a single dose of BCNU. No increase in median survival was noted, but there was a significant increase in the number of long-term survivors, which would be expected if a log or partial log of cell kill follows the 3–4-log cell kill achieved with BCNU alone. This result suggested that 5-FU might be effective used in combination with BCNU in the setting of a clinical trial.

In this study, 5-FU was administered by infusion over 3 days; infusions are less toxic than intermittent or daily injections (5,13) and a 3-day period was considered sufficient to expose all cycling tumor cells to the drug.[6] From the results of animal experiments, Hoshino extrapolated the repopulation kinetics of ic 9L tumor cells to human brain tumor cells.[6] Using his data, we concluded that the optimal time to administer 5-FU was 2 weeks after BCNU treatment.

MATERIALS AND METHODS

Patient Selection

Patients were accrued sequentially from the Brain Tumor Chemotherapy Service, Department of Neurological Surgery, University of California, San Francisco. All patients had recurrent malignant brain tumors documented by angiogram, radionuclide (RN) brain scan, computerized tomographic (CT) brain scan, and clinical neurologic examination. The eligibility requirements were unequivocal deterioration ascribable to tumor regrowth, no surgery for at least 2 months, no radiotherapy for at least 3 months, no chemotherapy for at least 6 weeks, and normal results of a cbc, platelet count, SMA-12 panel, urinalysis, and prothrombin time. Informed consent was obtained from each patient (and/or responsible next-of-kin) before therapy was begun.

Therapy

The original dose schedule included BCNU at a dose of 120 mg/m^2 iv (1 day) followed by 5-FU at a dose of 600 mg/m^2 iv (3 days), with courses repeated every 4 weeks. Because toxicity did not occur at these levels, the doses were escalated to 180 mg/m^2 of BCNU iv (Day 1) and 1 g/m^2/day of 5-FU iv (Days 15–17). Courses were repeated every 6 weeks, rather than every 4 weeks, to allow for bone marrow recovery from the higher dose of BCNU. Toxicity was carefully monitored. BCNU was decreased in patients experiencing severe marrow toxic reactions and then escalated in later courses to levels that could be tolerated by the patient. Patients who had no bone marrow toxic reactions had subsequent doses of BCNU escalated to even higher than 180 mg/m^2. The dose of 5-FU remained constant during all courses of therapy.

Glucocorticoid doses were adjusted as indicated to improve the level of neurologic function and to reduce headache and nausea. Because improvement caused by steroids can mimic response to cytotoxic therapy, doses were increased only when they were required to offset marked clinical deterioration. Attempts were made to reduce the steroid dose every 6 weeks if the patient's condition was stable or improving. Anticonvulsants and antiemetics were used when medically indicated.

Evaluation of Therapeutic Response

Thirty-seven patients were treated with BCNU-5-FU. Eight patients were unevaluable because their condition rapidly deteriorated or they died before receiving two courses of therapy. Twenty-nine patients had recurrent malignant gliomas (glioblastoma multiforme, anaplastic astrocytoma, gemistocytic astrocytoma, malignant glioma, or mixed malignant glioma), and comprised the study group for statistical analysis.

Effects of this combination therapy were evaluated from results of a neurologic examination and RN and CT scans (14). These tests were performed every 6 weeks, and the results were compared to the results of the preceding tests.

Response was defined as an improvement in at least two of the three tests, provided that the patient was receiving a stable or decreasing dose of steroids. *Progression* was defined as a deterioration in at least two of the three tests, provided that the patient had not had his/her dose of steroids decreased since the last course of therapy. *Stable disease* was defined as no change in the three tests, or improvement in only one test, or deterioration in

[6]Hoshino T. Personal communication, 1976.

TABLE 1.—Response by tumor type

Tumor type	No. of patients entered	No. of patients with—			No. of unevaluable patients
		Response	Stable disease	Progression	
Glioblastoma multiforme	7	1	2	1	3
Anaplastic astrocytoma	7	1	3	1	2
Recurrent low-grade astrocytoma*	14	6	8	0	0
Malignant glioma	7	0	1	3	3
Gemistocytic astrocytoma	1	0	1	0	0
Mixed malignant glioma	1	1	0	0	0
Totals	37	9	15	5	8

*Malignant at time of entry in this study.

only one test. Again, the patient had to be receiving a stable or decreasing dose of steroids.

RESULTS

Statistical analysis was based on 29 evaluable patients. Table 1 shows response by tumor type for all patients in the study: nine of 29 (31%) patients had a response to therapy, 15 of 29 (52%) had stable disease, and five of 29 (17%) showed progression.

Table 2 shows the duration of response and stable disease. Fourteen patients had an initial biopsy diagnosis of low-grade (grades I–II) glioma. At the time of this treatment, however, these patients showed both clinical and neurodiagnostic evidence of tumor recurrence and malignant transformation.

Using the Kaplan-Meier technique for nonparametric estimation from incomplete observations and the Gehan analysis of censored data (fig 1) (15,16), the time to tumor progression for the response and stable disease groups was found to differ statistically ($P < 0.01$). The estimated median time to progression was 27 weeks for all patients, 34 weeks for the response group, and 25 weeks for the stable group.

Figure 2 shows the mean (± 1 SD) toxicity levels for patients with leukopenia and thrombocytopenia who had toxic reactions to BCNU.

The bar graph in figure 3 shows the average BCNU dose (mg/m²/week) for patients with response and stable disease. T tests for each course demonstrated no significant difference between the two groups. However, F tests of equality of regression lines for five courses demonstrated a difference between the two groups ($P < 0.005$), which shows that response is dependent upon the highest possible dose of BCNU. Unfortunately, a patient who

TABLE 2.—Duration of response and stable disease

Tumor type	Duration (wks) of—	
	Response	Stable disease
Glioblastoma multiforme	27†	11,† 36
Anaplastic astrocytoma	32	11, 13, 32
Recurrent low-grade astrocytoma*	25, 26, 42, 52, 54, 66	17, 19, 21, 25, 29, 34, 43, 87†
Malignant glioma		45
Gemistocytic astrocytoma		12
Mixed malignant glioma	28	

*Malignant at time of entry in this study.
†Censored: patients died or were removed from study for reasons other than disease progression.

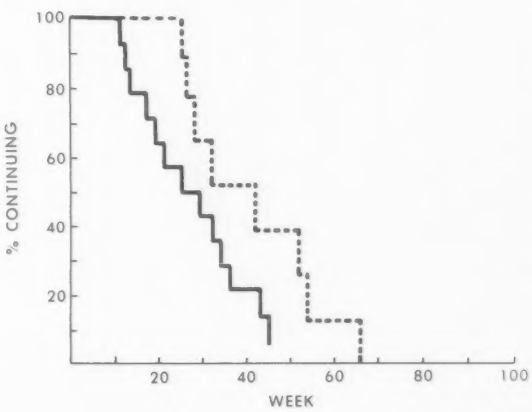
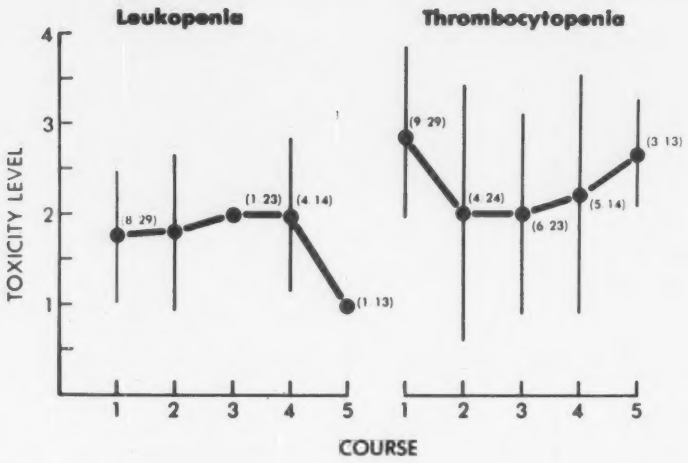


FIGURE 1.—Kaplan-Meier representation of BCNU-5-FU patients with response (dotted line) and stable disease (solid line); the 2 groups are different ($P < 0.01$) by the Gehan analysis (ref 15).



Toxicity Level	Leukopenia (Nadir WBC)	Thrombocytopenia (Nadir Platelets)
0	≥4000/mm^3	≥125,000/mm^3
1	3000-3999	100,000-124,999
2	2000-2999	75,000- 99,999
3	1500-1999	50,000- 74,999
4	<1500	< 50,000

FIGURE 2.—Mean toxicity levels per course of therapy (± 1 SD). Numerator = No. of patients who showed toxicity and denominator = No. of patients continuing to receive therapy.

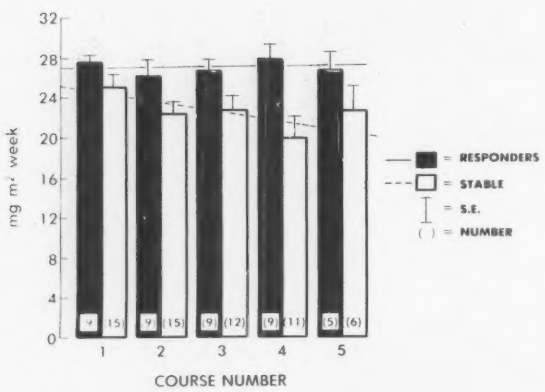


FIGURE 3.—Relationship of BCNU dose to response. The average BCNU dose/wk/course was computed for patients with response and stable disease; solid line and ■ = the response group and dashed line and □ = the stable disease group. Nos. in parentheses = No. of patients in each group. The error bars represent 1 SE. A linear regression analysis of the average dose of BCNU/wk/course vs course number shows a difference between the 2 groups (F test, $P < 0.005$), indicating that the stable disease patients tolerated lower doses of BCNU than the responding patients.

manifests dose-limiting bone marrow toxic effects is less likely to respond to therapy.

DISCUSSION

The goal of this study was to determine if a CCS drug used in combination with BCNU would provide more effective treatment of malignant brain tumors than either BCNU alone or BCNU used in combination with other drugs.

Response to therapy was evaluated with a neurologic examination and RN and CT scans. The overall activity level of patients was evaluated by the Karnofsky performance scale. Patients who responded to therapy had pretreatment Karnofsky ratings in the 70%–80% range; those who remained stable or had rapid disease deterioration had pretreatment Karnofsky ratings in the 50%–60% range. The response of a patient to therapy might be affected by his/her activity level at the beginning of treatment. This is substantiated by the fact that neurologic function is closely associated with tumor size. One half of the patients with response to therapy or disease stabilization improved in Karnofsky rating by 10%–20%, while all patients with

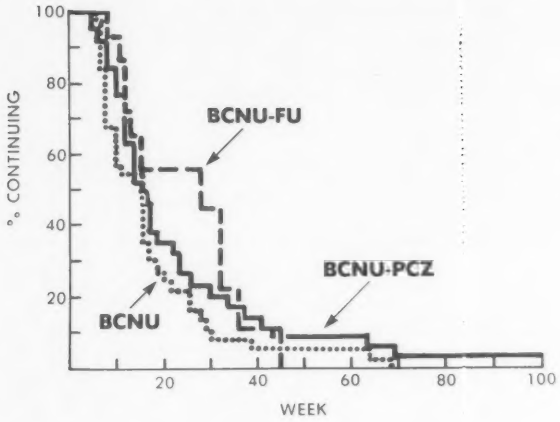


FIGURE 4.—Comparative Kaplan-Meier plots of time to tumor progression for BCNU (dotted line), BCNU-PCZ (solid line), and BCNU-5-FU (dashed line) for patients with glioblastoma, anaplastic astrocytoma, or malignant glioma. For the 3 groups the No. of patients = 37, 38, and 15, respectively. BCNU-5-FU was superior to BCNU alone ($P < 0.05$) but not superior to BCNU-PCZ ($P = 0.3$) by Gehan analysis (ref 15).

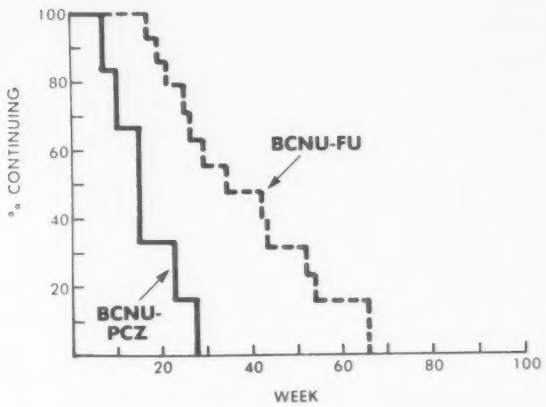


FIGURE 5.—Comparative Kaplan-Meier plots of time to tumor progression for BCNU-PCZ (solid line) and BCNU-5-FU (dashed line) for patients with original diagnoses of "low-grade glioma" who subsequently had recurrence with the appearance of "malignant glioma." For these groups the No. of patients = 14 and 6, respectively. BCNU-5-FU was superior to BCNU-PCZ ($P < 0.005$) by Gehan analysis (ref 15).

progression showed a 10%–20% decrease in Karnofsky ratings.

In previous studies, response was evaluated with two tests, the clinical neurologic examination and the RN scan. Response or progression was documented by improvement or deterioration in both tests. For this study, the CT scan was added to the tests. Response or progression was documented by improvement or deterioration in any two of the three tests. RN and CT scans can detect tumors in "silent areas" of the brain and often show tumor progression before clinical deterioration is evident. Thus, it is very likely that disease progression was detected earlier in this study than it was in previous studies; if the former criteria had been utilized here, time to progression could have been longer for some patients. With these considerations in mind and the realization that even historical series within the same institution may be biased, the time to progression for this study was compared to our previous studies of BCNU alone (9) and BCNU-procarbazine (PCZ) combination therapy (17).

Figure 4 shows the plots for patients with glioblastoma multiforme, anaplastic astrocytoma, and malignant glioma treated with BCNU (1,9), BCNU-PCZ (1,17), and BCNU-5-FU. None of the patients originally diagnosed as having low-grade glioma who subsequently developed malignant changes was included in this analysis; these patients are evaluated separately in figure 5. In figure 4, the BCNU-alone patients had a median of 12 weeks before tumor progression, the BCNU-PCZ group had a median of 15 weeks, and the BCNU-5-FU group had a median of 22.5 weeks.

There were a significant number of patients with initial diagnoses of low-grade glioma in the BCNU-5-FU study (14 of 29 patients), less in the BCNU-PCZ study (six of 44 patients), and none in the BCNU-alone study. When the first two studies were compared, a marked difference was found; the medians were 32 and 12 weeks, respectively, for time to tumor progression.

BCNU-5-FU was more easily tolerated by patients than BCNU-PCZ. PCZ not only compounds bone marrow toxic effects but also causes malaise and gastrointestinal toxic effects. The toxicity patterns found in this study and the observed relationship of BCNU dose to response suggest that BCNU should be initiated at a dose of 200 mg/m^2 in future clinical trials. To achieve greater cell kill, two courses of a CCS agent could be administered during each cycle. Drugs such as hydroxyurea or ftorafur, a depot form of 5-FU that is not myelosuppressive, might be used for ease of administration and convenience to the patient.

Alternatively, Mulder et al found that 5-FU administered prior to CCNU was superior to CCNU followed by 5-FU against Lewis lung and C22LR osteosarcoma tumors (18). They explained this observation by arguing that 5-FU synchronized tumor cells, which allowed CCNU to act more effectively against them. Although it has been difficult to

achieve cell synchronization in human solid tumors, the approach of Mulder and co-workers might be considered for brain tumors.

Given the less severe toxicity of this therapy, and the statistical significance of comparisons of the time to progression, BCNU-5-FU therapy demonstrates better control of brain tumors. We feel this combination therapy will be a valuable addition to chemotherapy protocols used to control brain tumors.

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Simulation Experiments of Tumor Measurement in Clinical Trials [1,2]

Dankward Kodlin* and Isidore Cohn, Jr [3,4]

SUMMARY

A simple model of tumor growth involving stochastic growth factors as well as measurement errors is explored in the context of a simulated "clinical trial" comparing two treatments. The model shows how treatment effects on tumor growth translate themselves into "improvement rates" derived from measurement on diameters. Furthermore, it shows that treatment contrasts of considerable magnitude (such as doubling of mass or volume versus shrinkage to one half) may escape recognition in clinical trials. A number of "power" tables are presented that provide the probability of making correct judgments of treatment effects. The tables indicate that the actual power in trials of this nature is in satisfactory agreement with that claimed in the standard power tables which are currently used in guiding trial strategy based on improvement rates.

[Cancer Treat Rep 62:2077–2083, 1978]

Within the last few years, clinical trials performed to test the effect of various therapeutic agents on tumors have adopted a procedure which is designed to evaluate rather rapidly at least the short-term effects of treatment; concentrating on patients with "measurable" disease, a palpable or visible lesion is measured at the beginning and at the end of treatment. If the measurements indicate a certain reduction in tumor size, the patient is called improved. Such "improvement rates," in a randomized experiment typically involving two different treatments, then form the basis on which to judge the relative merits of the treatments.

Since the strict adherence to a treatment protocol often ends at the end of a "trial" (the surviving patient then being treated further at the clinician's discretion), survival time information becomes less suitable for judgment with potential treatment effects being confounded by subsequent therapeutic efforts. Thus, the short-term evaluation of tumor progression has become, in fact, the mainstay of a highly organized experimental enterprise. It is, therefore, of considerable interest to assess in some way the errors inherent in the measurement procedures and to investigate the effects of these errors on the improvement rates and on the statistical conclusions derived from these rates.

In the absence of special studies to assess the relation between measured quantities and true tumor size (which probably would require special investigations on autopsies), Moertel and Hanley (1) have approached the problem recently by presenting a set of "phantom" tumors, spheres of known size under foam rubber covering, to a group of oncologists experienced in the measurement task. Under these ideal conditions, it appears that the (single) diameter of these structures is being estimated with an error of the order of 12%. That is to say, on the average, measurements deviate from the true diameter by about 12% of that diameter, disregarding direction. The consequence of this becomes clear when these data are translated into the setting of a trial. Adopting an "area" measurement, in their case simply the square of the measured diameter, the authors have demonstrated that about 7%–8% of tumors that have remained per-

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[4]Since power estimates based on 100 replications are subject to noticeable sampling fluctuations, runs with 1000 replications have been made whenever necessary. We thank Cassis Henry for her assistance in making such runs on high-speed equipment. We have benefited from critiques provided by Drs. John Crowley and Hans Ury.

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fectly unaltered between two occasions of measurement, such as the beginning and end of a trial, would be judged to have "improved" if improvement were defined as a reduction to at least one half of the initial area. On the other hand, a "genuine" reduction, say to 40% of the initial area, would be correctly judged only in about 73% of patients in whom it would occur. In other words, there appears to be a "sensitivity" of 73% and a "specificity" of 92%–93% in this diagnostic tool, based on the exploration with the phantom. These data would suggest that the technique is not as precise as certain laboratory tests in other fields which can reach both sensitivity and specificity of > 95%.

Returning to Moertel and Hanley's discussion of their findings, the argument is made that, while in clinical practice these misclassifications are likely to be even larger than those based on the measurement error rate of 12% for the diameter of a phantom, natural tumor growth, ie, uninhibited by treatment, if assumed to be of the order of twofold by volume within 2 months, would surely decrease the frequency with which a false-positive, a reduction in area by as much as 50%, would be asserted. Yet, if the two radii are r_1 and r_2 on the first and second occasions, then a 2.0-fold growth factor by volume $(r_2/r_1)^3 = 2$, corresponds to a 1.6-fold growth factor by area $(r_2/r_1)^{2/3} = 2^{2/3} = 1.6$, and to a 1.26-fold growth factor by diameter $(r_2/r_1)^{1/3} = 2^{1/3} = 1.26$.

Thus, one wonders how often a growth factor of this magnitude will be misjudged, with measurement errors of 12% on the diameter. Indeed, looking at the phantom tumors Nos. 10 and 11 in table 1 of these authors (1), which have diameters of 7.5 and 8.6 cm, and thus could stand for a tumor growing by a factor of 1.26 on the diameter, we note that one of 16 investigators read these as 6.5 and 4 cm and thereby concluded that the tumor did shrink > 50% ($4^2/6.5^2 = 0.38$). Thus, improvement rates of 6% (one of 16 patients) would be obtained from treatments which lead to a doubling of tumor in 2 months.

Certainly, an anecdotal observation of this kind does not give sufficient insight to assess the performance characteristics of full-fledged clinical trials because they are affected not only by the noise of such measurement errors but also by the unexplored vagaries of tumor growth which are bound to be stochastic in nature so that, while the growth factor of 2, for instance, may apply as an average value to a group of patients, it will show genuine variation between patients and between the two unequal diameters that present themselves for actual measurement. It would have been extremely awkward to attempt a simulation of this additional source of variability by carrying out phantom experiments that incorporate such features and to obtain sufficient replications that would assess the compound effect on the trial outcome. We have, therefore, carried out full-fledged simulation experiments involving also "complete" trials, that is, contrasts of "observed" improvement rates for two "treatments" affecting the tumor growth in a variety of ways.

In this report we present our conclusions based on > 500,000 imaginary patients, whose tumors were created and measured by generating random numbers on a small computer. The analysis of this material (straightforward computations of improvement rates and chi-square test statistics) was carried out on the same machine. While we thus could deal with large numbers, our individual trials were kept well within the bounds of clinical reality, limiting the "trial size" (that is, the number of patients per treatment) to values as low as 10 and never exceeding 40.

TABLE 1.—Outcome of simulated tumor growth observations on 10 patients

	First occasion					Second occasion					
	True diameter		Diameter as read		Area	True diameter		Diameter as read		Area	Patient improved?* (area shrunk ≥ 50%)
Patient No.	No. 1	No. 2	No. 1	No. 2	as read	No. 1	No. 2	No. 1	No. 2	as read	
1	3.3	1.3	5.5	1.7	9.2	3.0	2.2	3.0	2.2	6.8	No
2	5.0	7.2	7.8	4.0	31.6	5.0	12.8	3.9	13.5	52.9	No
3	4.9	4.7	5.9	3.6	21.3	9.2	7.5	5.3	3.4	18.1	No
4	8.6	1.4	8.8	1.5	13.5	7.0	1.4	2.6	1.0	9.9	No
5	7.2	6.6	8.3	5.7	47.2	8.6	8.4	5.0	4.0	20.0	Yes
6	5.3	4.7	5.6	4.2	23.9	12.0	12.6	16.6	4.2	70.2	No
7	8.0	4.1	9.6	5.6	54.0	20.6	5.6	22.8	5.3	120.3	No
8	9.9	9.8	8.3	9.7	80.5	9.8	18.4	10.0	11.8	117.7	No
9	3.8	5.9	1.4	5.2	7.5	5.3	5.4	3.9	5.4	21.0	No
10	4.3	7.8	5.0	8.4	42.0	7.1	5.7	7.2	1.0	7.2	Yes

*Improvement rate = 20%.

METHODS

As illustrated in table 1, line 1, we start by drawing a random number between 1 and 10 cm from a uniform distribution, representing the first diameter. The second diameter is drawn independently from the same distribution. A third drawing, this time from a logarithmic distribution (*see* Appendix, No. 2), creates a value, say e_1, between 0 and 1. The first diameter, say d_1, is then taken "as read" to be

$$m_1 = d_1(1 \pm e_1),$$

the quantity e being either subtracted or added, depending on a random event which we let occur with a probability of 1/2. The imaginary reader is thus unbiased, on the average over-reading as often as under-reading. A fourth drawing establishes similarly the second diameter "as read":

$$m_2 = d_2(1 \pm e_2)$$

and the product m_1m_2 becomes the area "as read."

The growth of the tumor is now simulated by the random drawing from a Weibull distribution (*see* Appendix, No. 3) which can take on values between 0 and ∞. For patients subject to a twofold volume growth factor, we sample from a diameter growth factor distribution with a mean of 1.26, this being the expected growth factor operating on the diameter. If the value drawn is g_1, the original diameter d_1 becomes

$$d'_1 = d_1g_1.$$

Evidently, if $g < 1$ is drawn, the diameter will shrink, while it increases upon drawing $g > 1$.

Repeating the operation a second time for d_2 and then drawing measurement errors for d'_1 and d'_2, we obtain the new area, say $m'_1m'_2$, as the right portion of table 1 indicates. For patient 1, in conclusion, there is no improvement, although the area on the second occasion is smaller than that on the first.

These operations are repeated for the remaining patients, each time drawing new random numbers as described. Note the variety of areas created in this fashion, reflecting the different kinds of measurable tumors presented by these patients.

Assigning the ten patients to a given treatment, A, we get the result of a 20% improvement rate while the tumor stochastically has doubled its volume. For treatment B, a similar round of drawings is performed. Before coming to the growth factor we must, of course, decide if a relatively effective treatment is to be simulated, in which case the distribution of growth factors has to be altered correspondingly. Having decided that B should be equivalent to A (allowing a doubling in tumor volume), we keep the growth factor distribution unaltered. Suppose the outcome on B would give in our example an improvement rate of 10% only. The contrast in improvement rates would then be 20% versus 10% and a $\chi^2 < 3.84$ would result so that one would (correctly) accept the "null hypothesis" of "no difference." Conclusions of this nature complete a single simulated trial. To empirically assess the vagaries of chance operating within this model, a trial of this kind is then repeated 100 times, as described in the following section. A few additional mathematic details are listed in the Appendix. Table 1 may be used to assess the magnitude of the measurement error. The observed average relative deviation[5] is 27%, clearly in excess of the 12% estimated on the phantom and in good agreement with the value of 25% expected from the model (*see* Appendix, No. 2). Note also that while there is a general tendency for the tumor to grow, on occasion a diameter will shrink (eg, diameter No. 1 of patient 4).

RESULTS

Standard model.—Table 2 gives the results of a systematic exploration of our model. Line 1, assuming volume growth factors of 2.0 for both treatment A and B, shows that the model leads to average improvement rates of 25% in both groups and indicates that if the null hypothesis, "no difference in treatments," is true, the χ^2 tests will not be misleading. Indeed, rather than getting the customary 5% of falsely positive χ^2 values (> 3.84), we see in 100 actual repetitions of these trials, for various sample sizes, that on the average, only about 2% of such "critical" χ^2 values are obtained. The χ^2 test thus appears to be somewhat conservative if the null hypothesis is true. This feature may simply be due to the "correction for continuity" which we have applied throughout. Lines 2–5 of table 2 indicate the trial's ability to pick up, as "statistically significant," differences between treatments, as reflected by differences in growth factors between treatments. Line 3, for instance, assumes that while under treatment A the volume doubles, under treatment B the volume shrinks to one half of its initial size, and it then shows that such an effect would be recognized only in 48% of trials with 30 patients in each treatment. It is clear from this table that only if B is a fairly effective treatment, reducing the tumor volume to 50% of its initial size and being in

[5] $\frac{1}{n}\sum|(m_i - d_i)/d_i| = |e_i|$, averaged over all measurements.

TABLE 2.—Improvement rates and power for various treatment contrasts*

Tumor growth characteristics and resulting improvement rate				% of trials in which a significant χ^2 (> 3.84) would be obtained (power)			
Volume growth factor		Improvement rates (averaged over all trials)		No. of patients/treatment			
Treatment A	Treatment B	Treatment A	Treatment B	10	20	30	40
2	2	0.25	0.25	1	2	2	2
2	1	0.25	0.38	1	5	10	16
2	0.5	0.25	0.52	10	30	48	70
2	0.1	0.25	0.84	80	98	100	100
2	0.01	0.25	0.99	99	100	100	100
1	1	0.38	0.38	1	0	3	2
1	0.5	0.38	0.52	4	8	18	24
1	0.1	0.38	0.84	31	84	95	99
1	0.01	0.38	0.99	79	100	100	100

* For assumptions on growth characteristics and measurement errors *see* test.

TABLE 3.—Rerun of trials simulated in table 2, eliminating measurement errors

Tumor growth characteristics and resulting improvement rate				% of trials in which a significant χ^2 (> 3.84) would be obtained (power)			
Volume growth factor		Improvement rates		No. of patients/treatment			
Treatment A	Treatment B	Treatment A	Treatment B	10	20	30	40
2	2	0.17	0.17	0	0	2	2
2	1	0.17	0.30	1	8	11	19
2	0.5	0.17	0.50	12	51	65	81
2	0.1	0.17	0.94	93	100	100	100
2	0.01	0.17	1.00	100	100	100	100
1	1	0.30	0.30	0	5	3	3
1	0.5	0.30	0.50	4	13	19	32
1	0.1	0.30	0.94	73	100	100	100
1	0.01	0.30	1.00	93	100	100	100

addition compared with an ineffective treatment, does one obtain some assurance (70%) of recognizing the effect with 40 patients per treatment. If, on the other hand, A is already effective to the extent of inhibiting tumor growth completely (volume growth factor = 1), the superiority of B will more often be missed (76%) than recognized (24%). Only if B is dramatically effective, shrinking the tumor size to one tenth of its original value, can one be certain to pick it up as significantly better than A, with 40 patients per drug, the power now being 99%.

Eliminating measurement error.—The difficulty in recognizing effective treatments just demonstrated is only partially due to measurement errors. The erratic nature of tumor growth is bound to limit power even in the absence of any measurement error. The question is, then, how far one could improve the power of recognition if measurement error could be completely eliminated. Table 3 presents our findings from trials in which the tumor areas have been measured precisely. While the power is consistently improved, it is somewhat disappointing to note that no dramatic progress is being made. For instance, in line 2, a trial with 40 patients would recognize treatment B as superior to treatment A in 19% of trials, instead of 16% with measurement error, suggesting that an attempt to improve the latter would not be very rewarding.

Modifying growth factor independence.—In the preceding experiments, the growth factors for the two diameters were obtained independently as described in the Methods section. Thus, one diameter of a given tumor could shrink while the other grew. Variants of our standard model are explored in table 4, with growth factors 2 and 0.5 for treatments

TABLE 4.—Effects on power of measurement error, No., and way of using diameters and growth factor characteristics*

Line No.	Measurement error	No. and relation of diameters measured	Growth characteristics of diameters	Tumor description	Improvement rates		% of trials in which a significant χ^2 (> 3.84) would be obtained (power)			
							No. of patients/treatment			
					Treatment A	Treatment B	10	20	30	40
1	Yes	2, independent	Independent	Eccentric, changing shape	0.25	0.52	10	30	48	70
2	No	2, independent	Independent	Eccentric, changing shape	0.17	0.50	12	51	65	81
3	Yes	2, independent	Identical	Eccentric, keeping shape	0.26	0.49	7	21	36	48
4	Yes	2, identical	Identical	Round, keeping shape	0.26	0.50	6	21	32	45
5	No	2, identical	Identical	Round, keeping shape	0.22	0.46	6	30	39	55
6	Yes	1, —	—	—	0.11	0.22	1	6	10	16
7	No	1, —	—	—	0.12	0.27	3	15	20	23
8	Yes	2, independent; 3rd power of geometric mean	Independent	Eccentric, changing shape	0.29	0.60	12	33	58	74

*This table considers a volume growth factor of 2 for treatment A and 0.5 for treatment B.

A and B, respectively. The first two lines repeat the corresponding entries of tables 2 and 3. Line 3, to be compared with line 1, clearly indicates that if one deals with "eccentric tumors keeping shape" the power is markedly affected. In other words, on such tumors, treatment evaluation is more difficult than on tumors changing shape.

Simulation of round tumors.—Round tumors keeping shape [apparently the prevailing pattern in the lung (3)] are considered on line 4 of table 4. They behave like eccentric tumors keeping shape (line 4 versus line 3) and, once again, elimination of measurement error does not give great gain in power (line 5 versus line 4).

Changing the improvement index.—Since the choice of an area measurement as well as the definition of improvement are quite arbitrary, one may ask if other choices would improve the discrimination between treatments A and B. For instance, one might restrict measurement to one diameter and define improvement as reduction of the diameter by at least one half. As lines 6 and 7 (table 4) show, this practice leads to smaller differences in improvement rates and a remarkable loss of power. On the other hand, if one would choose improvement to mean at least arrest of growth, theoretically expected improvement rates would be 39% and 71% for A and B, respectively, a difference of 32% and a result as good as that seen in line 2. Indeed, it can be shown that such a definition of improvement is very near the optimum (*see* Appendix).

If area measurements are available, the simple device of evaluating improvement as a 50% reduction in the third power of the geometric mean of the two diameters might be advantageous, as a comparison of line 8 with line 1 suggests. These observations and those in the preceding paragraph simply indicate that alternatives to current practices of defining improvement may be equally effective or better in discriminating between treatments and one might like to explore them also in the analysis of genuine trial data.

DISCUSSION

The following conclusions can be drawn from the findings of the preceding section.

A. If measurement errors on the tumor diameter are increased beyond those observed under ideal phantom conditions, a 25% improvement rate would be read under conditions of tumor growth that amounts to a doubling in tumor volume in 2 months. If one follows Moertel and Hanley (1) in adopting an estimate of Steel (4) leading to such a doubling time as a global value for malignancies, such improvement rates cannot be interpreted as treatment-induced.

B. Quite similar improvement rates will appear if the measurement error is completely eliminated and the remaining source of variation, namely, that in diameter growth, is retained. More importantly, the power of such trials is visibly affected by the measurement errors, but if the latter are completely eliminated the power does not remarkably improve.

C. In genuine trials, alternatives to the standard improvement criterion (50% area reduction) should be explored since they might give better discrimination between treatments.

It has been claimed quite generally (5–10) that measurement errors lead to a reduction in power. Our findings demonstrate this general principle.

The question remains whether the standard power tables, giving the necessary sample sizes for given differences in improvement rates, apply to our models. These tables turn out to be applicable. For instance, in line 3 of table 2 we find the expected improvement rates of 0.25 and 0.52. For a difference of this nature, a standard table (11) would claim that (with $\alpha = 0.02$) a power of 50% would be obtained with 35 patients. Our trials show that such a power is obtained with slightly > 30 patients. Similarly, in line 4 of table 4, a difference in improvement rates such as 0.26 versus 0.50 would be claimed, from a regular power table, to be recognizable in 50% of trials using 45 patients, while we get a power of 45% with 40 patients. Once more, for a contrast in improvement rates such as 0.38 versus 0.84 (line 8 of table 2), the standard table would promise a power of 95% with 38 patients while we find that 30 patients are necessary. Thus, power tables based on improvement rates appear to be realistic.

The proposition of any model invariably raises the question of its validity. Are the few assumptions realistic enough to display the essential features of the process of tumor growth and its inhibition? One can think of numerous alternatives to be explored: changing the growth factor distribution, complicating the mode of growth control, introducing additional variability between patients, increasing measurement errors, etc. For many such variants one can, of course, expect the direction of their impact upon the power of clinical trials. The limited exploration reported above will surely not give more than a conjectural answer to the question of what kind of treatment effects are likely to be uncovered or missed by the current clinical experimentation. The answer may nevertheless be helpful in focusing on the basic issue: should one concentrate on the improvement in recognizing "moderate" effects (essentially by putting more patients on a given trial, thus reducing the number of treatments coming to trial) or should much more emphasis be given to the alternative, a "screening for dramatic effects," in which a much larger number of treatments and combinations could be evaluated with the same resources while one would fail to recognize moderate progress if it exists?

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APPENDIX

1. Diameters, d, are sampled from a uniform distribution by transforming the random variable r(0,1), obtained from the random number generator, into $d = 9r + 1$.

2. Measurement errors, e, are sampled from the distribution $f(e) = -\ln e$, by taking $e = r_1 r_2$, where r_1 and r_2 are r(0,1), independently drawn. The mean of this distribution is 0.25 and a sample of m values such as given in table 1 should, therefore, differ by 25% from the corresponding d's, on the average.

3. Growth factors, g, are sampled from a 1-parameter Weibull distribution (2)

$$f(g) = \lambda g e^{-1/2\,\lambda g^2},$$

with mean $$\mu = \sqrt{\pi/2\lambda}, \qquad [1]$$

by transforming $$r(0,1) \text{ into } g = \sqrt{-(2/\lambda)\ln r}. \qquad [2]$$

A k-fold growth factor by volume implies a growth factor of $k^{1/3}$ by diameter.

Thus, $\mu = k^{1/3}$ and using [1], the transformation [2] becomes
$g = 2k^{1/3}\sqrt{-(1/\pi)\ln r}$.

Note also $$\int_0^x f(g)\,dg = 1 - e^{-1/2\,\lambda x^2}.$$

Thus, for $k = 2$, implying $\lambda = 0.9895$,
$1 - e^{-1/2\,\lambda} = 0.39$ is the frequency of g-values ≤ 1

and $1 - e^{-1/2\,\lambda\,1/4} = 0.12$ is the frequency of g-values ≤ 0.5.
For $k = 0.5$, implying $\lambda = 2.4935$,
$1 - e^{-1/2\,\lambda\,1/4} = 0.27$ is the frequency of g-values ≤ 0.5.

Thus, improvement rates of 12% and 27% would be obtained for treatments A ($k = 2$) and B ($k = 0.5$) if only 1 diameter would be used and if improvement would be defined as shrinkage to at least one half of the original diameter. Note the complete agreement of line 7, table 4, with these expected rates. The maximal difference between improvement rates is obtained if

$$x = \sqrt{-[2/(\lambda_A - \lambda_B)]\ln \lambda_A/\lambda_B} = 1.108.$$

Thus, using only 1 diameter, an optimal definition of improvement would be diameter growth by < 10.8%, leading to improvement rates of 44% and 77% for A and B, respectively.

Therapeutic Potentiation of Nitrosoureas Using Chlorpromazine and Caffeine in the Treatment of Murine Tumors [1,2]

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SUMMARY

The therapeutic usefulness of chlorpromazine (CPZ) and caffeine (CAF) in combination with selected nitrosoureas was investigated in mice bearing L1210 leukemia, Lewis lung carcinoma, and B16 melanoma. We found that using BCNU with either CAF or CPZ was therapeutically superior to using either agent alone to treat mice bearing L1210 leukemia. Administering all three drugs in combination did not improve upon the therapeutic responses obtained with the two-drug combinations. In mice implanted with Lewis lung carcinoma or B16 melanoma, responses to treatment with the triple combination of methyl-CCNU, CAF, and CPZ suggested, but did not clearly establish, superiority over each two-drug combination or methyl-CCNU alone.

[Cancer Treat Rep 62:2085–2093, 1978]

The enhancement of the antileukemic activity of BCNU by various psychotropic drugs in combination with caffeine (CAF) was described by Cohen (1,2). He reported, for example, that chlorpromazine (CPZ) and CAF given with BCNU significantly increased the survival rate among mice bearing L1210 leukemia when compared to the survival of leukemic mice receiving BCNU alone or any of the two-drug combinations. CPZ alone has been reported to be mildly cytostatic against 37 sarcoma (3), a murine mastocytoma (4), and B16 melanoma (B16) (5); however, neither CPZ nor CAF cured any mice implanted ip with 10^5 L1210 cells when single-dose treatment was given 5 days after the implant (1). Recently, Driscoll et al (6) reported that multidose CPZ therapy (given every day on Days 1–9 or on Days 1, 5, and 9) of mice implanted ip with 10^5 L1210 cells resulted in only "inconsistent antitumor activity."

The purpose of the studies reported here was to attempt to confirm and extend Cohen's original observations. In addition to evaluating BCNU in combination with CAF and CPZ against L1210 leukemic mice, we sought to determine if a similar drug combination would be therapeutically potentiating against other murine tumors such as B16 and Lewis lung carcinoma (LL), which have been shown to be sensitive to nitrosourea alkylating agents (7–9).

MATERIALS AND METHODS

Mice.—C57BL/6, DBA/2, BDF_1, and CDF_1 mice of both sexes, 6–8 weeks old, were used in these experiments. Wayne Lab Blox (Allied Mills, Chicago, Ill) and water were provided ad libitum.

Tumors.—The B16 and LL tumors were maintained by serial sc passage in C57BL/6 mice. L1210 leukemia was maintained by serial passage of ascites cells in DBA/2 mice. All L1210 studies were initiated by implanting 10^5 or 10^6 (trypan blue-excluding) leukemia cells ip into CDF_1 mice and therapy was given on Day 5 after implantation only. A serial $\log_{10}$ dilution titration (down to one cell) of the donor L1210 cell suspensions used in each experiment was included to allow objective estimates of population doubling time and viable tumor cells surviving drug treatment (10). All LL studies

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TABLE 1.—Characterization of stage of disease progression in mice used in this study following implantation with L1210 leukemia, LL, and B16

Tumor implanted	Route	No. of cells	Treatment begun on Day:	Approximate tumor cell burden at initiation of therapy	MST (days) of untreated controls (dying mice only)*
L1210	ip	10^6	5	$> 10^9$	7.0
		10^5	5	$\geq 10^8$	8.0
LL	iv	10^6†	10	?	15.5–18.0
B16	iv	10^6‡	4	?	32
			14	?	30
	sc	10^4‡,§	2	?	36.5 (1 of 15 mice survived)
			6	?	41.0 (2 of 15 mice survived)

* All untreated control mice died of tumor except where indicated.
† About 5%–10% of the cells excluded trypan blue.
‡ About 1% of the cells excluded trypan blue.
§ Implanted sc with 10^7 ^{60}Co-irradiated B16 cells.

were initiated by implanting 10^6 monodispersed tumor cells (5%–10% excluded trypan blue) iv into BDF_1 mice and administering therapy on Day 10 after implantation only. B16 studies were begun by implanting BDF_1 mice either sc with 10^4 monodispersed tumor cells (approximately 1% excluded trypan blue) and treating them on Day 2 or 6 after implantation, or iv with 10^6 cells (approximately 1% excluded trypan blue) and treating them on Day 4 or 14 after implantation. When 10^4 B16 cells were implanted sc, they were mixed with approximately 10^7 lethally irradiated (^{60}Co) B16 "feeder" cells used to increase the otherwise poor tumorigenicity of the small inocula as described previously (11). These LL and B16 implants were chosen on the basis of historical data indicating them to be the smallest inocula capable of causing death in ≥ 90% of untreated control mice. A summary of the tumor implant information, as well as estimates of tumor body burden at initiation of therapy and the median survival time (MST) of untreated controls, is given in table 1.

Assessment of antitumor activity.—Evaluation of antitumor activity was based upon two parameters: (*a*) the percentage of long-term survivors ("cures") determined ≥ 110 days after implantation for mice implanted with B16, 80 days after implantation for LL-implanted mice, and 60 days after implantation for L1210-implanted mice; and (*b*) the percent increase in lifespan (ILS), T–C/C × 100, where "T" is the MST of the dying mice in a treated group and "C" is the MST of the untreated tumor-bearing control mice. Comparisons of chemotherapeutic activities were made on the basis of optimal therapeutic responses achieved at maximum-tolerated dose (MTD) levels (defined as not being lethal for > 10% of the drug-treated mice) unless otherwise specified.

Drug toxicity was judged by the mortality patterns of tumor-bearing mice during and following treatment in experiments involving LL and B16. Generally, mice dying during or up to 1 week after treatment, with or without tumors (determined by necropsy), were considered to be drug deaths. Lethal drug toxicity in L1210-bearing mice was assumed to be the same as in parallel groups of nonleukemic toxicity control mice treated with the same drug doses at the same time as the leukemic mice, or by necropsy of all drug-treated mice implanted with L1210 and observation made of the presence or absence of overt leukemia (ascites and/or splenomegaly).

Drugs.—The following drugs, obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, Md), were used in this study: BCNU, methyl-CCNU (meCCNU), and CAF. CPZ was obtained commercially. All drugs were administered ip except CAF, which was administered sc. Mice received their appropriate drug doses on a milligram per kilogram basis according to the average body weight of the group under treatment. When CPZ was given in combination with another drug or drugs, it was always injected 6 hours prior to the other drug(s). When CAF was given with BCNU or meCCNU, it was injected 10–15 minutes following the nitrosourea. These specific sequential drug administrations were chosen based upon the work of Cohen (1,2).

RESULTS

L1210 Leukemia

Three replicate experiments were performed in which mice were implanted ip with 10^5 L1210 cells

and treatment was given on Day 5 after the implant (fig 1), at which time their tumor cell burden was estimated (based upon calculated population doubling times of 0.31–0.37 day) to have been $\geq 10^8$ cells. The MST of untreated leukemic control mice in each experiment was 8 days, so the stage of disease progression on Day 5 after the implant may aptly be described as advanced.

We evaluated BCNU alone and in combination with CPZ and/or CAF. At the approximate MTD levels, neither CPZ (75 mg/kg) nor CAF (200 mg/kg) alone had any demonstrable antileukemic activity. However, two-drug combinations of BCNU plus either CPZ or CAF caused therapeutic responses that were superior to that found using BCNU alone at any dose level up to and including the MTD, 40 mg/kg. This therapeutic synergism was evident by both the increased cure rates and the greater ILSs occurring among dying mice.

For example, in the first experiment (fig 1A), the optimum response achieved with 40 mg/kg of BCNU alone was one of ten mice cured with a 75% ILS (lower doses of BCNU achieved a maximum of 131% ILS, but without any cures). In contrast, 30 mg/kg of BCNU plus either 150 mg/kg of CAF or 15 mg/kg of CPZ yielded eight of ten mice cured with a 212% ILS or six of ten mice cured with a 218% ILS, respectively. When all three drugs were used in combination, as many as eight of ten mice were cured (156% ILS), but this was not better than the optimal responses achieved with the previously described two-drug combinations. Several other two- and three-drug combinations involving BCNU and either, or both, CAF and CPZ (not illustrated) at MTD levels cured four to six of ten mice while causing 231%–268% ILSs among the dying mice.

In the second (fig 1B) and third (fig 1C) experiments, we observed the same relative therapeutic activities for each drug and drug combination that were found in the first experiment. That is, BCNU plus either CAF or CPZ was therapeutically superior to any of those drugs alone, and the use of all three drugs together did not improve upon the therapeutic responses obtained with the two-drug combinations. Therapeutic potentiation among mice receiving combination chemotherapy (as compared to BCNU alone) was evident from both the better cure rates and greater ILS values found among the dying mice.

As part of the last experiment, additional groups of mice were implanted ip with 10^6 L1210 cells and treatment was withheld until Day 5 after the implant when their tumor burden was estimated to have been $> 10^8$ L1210 cells. Evaluating all of the same regimens that were used in mice bearing the smaller tumor burden (10^5 L1210 cell implants), we failed to observe a meaningful therapeutic effect. BCNU alone caused a maximum response of only 28% ILS, and none of the combination chemotherapy regimens induced > 42% ILS (data not shown).

Lewis Lung Carcinoma

Mice implanted iv with 10^6 LL cells were treated with meCCNU alone, or meCCNU, CAF, and CPZ in various double- and triple-drug combinations on Day 10 after implantation (fig 2). Three essentially replicate experiments were performed. The combination of 15 mg/kg of CPZ plus 150 mg/kg of CAF was evaluated twice and was without therapeutic activity. A single dose of 24 mg/kg of meCCNU (the approximate MTD was 36 mg/kg) failed to cure any of the treated mice but yielded ILSs which varied from 35% to 67% in different experiments. The addition of 150 mg/kg of CAF to this dose of meCCNU failed, in each experiment, to potentiate the therapeutic responses obtained with meCCNU alone. Combination chemotherapy using 24 mg/kg of meCCNU plus 15 mg/kg of CPZ also failed to cure any mice and yielded ILSs of between 58% and 72%. These responses represented only minimal and inconsistent improvement in MST compared to those achieved with 24 mg/kg of meCCNU alone.

In contrast to the responses obtained with the various two-drug combinations, the application of all three drugs in combination resulted in therapeutic responses that were comparable to or greater than those obtained with only the most active component, meCCNU. In the first experiment (fig 2A), treatment with 24 mg/kg of meCCNU alone caused a 50% ILS but no cures, whereas the addition of 15 mg/kg of CPZ and 100 or 150 mg/kg of CAF to the treatment regimen not only resulted in 77%–94% ILSs among dying mice but also one of ten mice were cured in each of the two combination groups. However, the greater percent ILS occurred with the drug combination which included 150 mg/kg of CAF and which was judged to have caused two of ten drug deaths. These therapeutic responses were comparable to that obtained using a single dose of 36 mg/kg of meCCNU: 116% ILS but no cures.

In a replicate experiment (fig 2B), neither 24 nor 36 mg/kg of meCCNU caused therapeutic responses comparable to that obtained with 24 mg/kg of meCCNU plus 15 mg/kg of CPZ plus 150 mg/kg of CAF. Whereas 24 or 36 mg/kg of meCCNU alone, each of which appeared to have caused two of ten drug deaths, achieved 35% and 47% ILSs, respectively, the triple-drug combination described (an MTD) resulted in a 147% ILS. A similar therapeutic advantage was found in the third experiment (fig 2C) although it was not quite as marked. MeCCNU

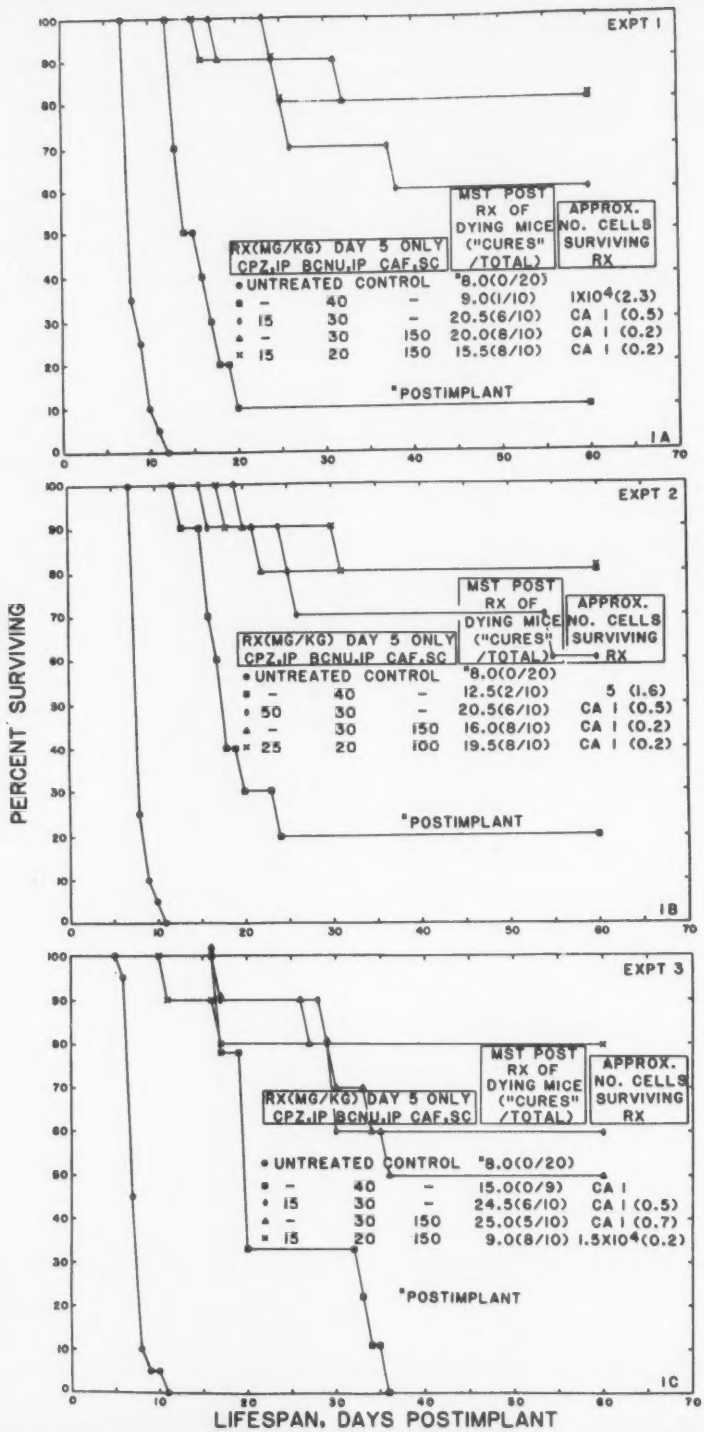
EXPT 1
RX(MG/KG) DAY 5 ONLY
CPZ,IP BCNU,IP CAF,SC
MST POST RX OF DYING MICE ("CURES" /TOTAL)
APPROX. NO. CELLS SURVIVING RX
•UNTREATED CONTROL *8.0(0/20)
■ – 40 – 9.0(1/10) 1X10⁴(2.3)
• 15 30 – 20.5(6/10) CA 1 (0.5)
▲ – 30 150 20.0(8/10) CA 1 (0.2)
× 15 20 150 15.5(8/10) CA 1 (0.2)
*POSTIMPLANT
1A
EXPT 2
RX(MG/KG) DAY 5 ONLY
CPZ,IP BCNU,IP CAF,SC
MST POST RX OF DYING MICE ("CURES" /TOTAL)
APPROX. NO. CELLS SURVIVING RX
•UNTREATED CONTROL *8.0(0/20)
■ – 40 – 12.5(2/10) 5 (1.6)
• 50 30 – 20.5(6/10) CA 1 (0.5)
▲ – 30 150 16.0(8/10) CA 1 (0.2)
× 25 20 100 19.5(8/10) CA 1 (0.2)
*POSTIMPLANT
1B
EXPT 3
RX(MG/KG) DAY 5 ONLY
CPZ,IP BCNU,IP CAF,SC
MST POST RX OF DYING MICE ("CURES" /TOTAL)
APPROX. NO. CELLS SURVIVING RX
•UNTREATED CONTROL *8.0(0/20)
■ – 40 – 15.0(0/9) CA 1
• 15 30 – 24.5(6/10) CA 1 (0.5)
▲ – 30 150 25.0(5/10) CA 1 (0.7)
× 15 20 150 9.0(8/10) 1.5X10⁴(0.2)
*POSTIMPLANT
1C
PERCENT SURVIVING
LIFESPAN, DAYS POSTIMPLANT

FIGURE 1.—Therapeutic potentiation of BCNU by CPZ and CAF in mice implanted ip with 10^5 L1210 cells (10 mice/group except untreated controls, 20 mice). Estimates of the number of leukemic cells surviving treatment were based upon the proportion of mice surviving (if any) and/or the MST (days) of the dying mice (ref 10).

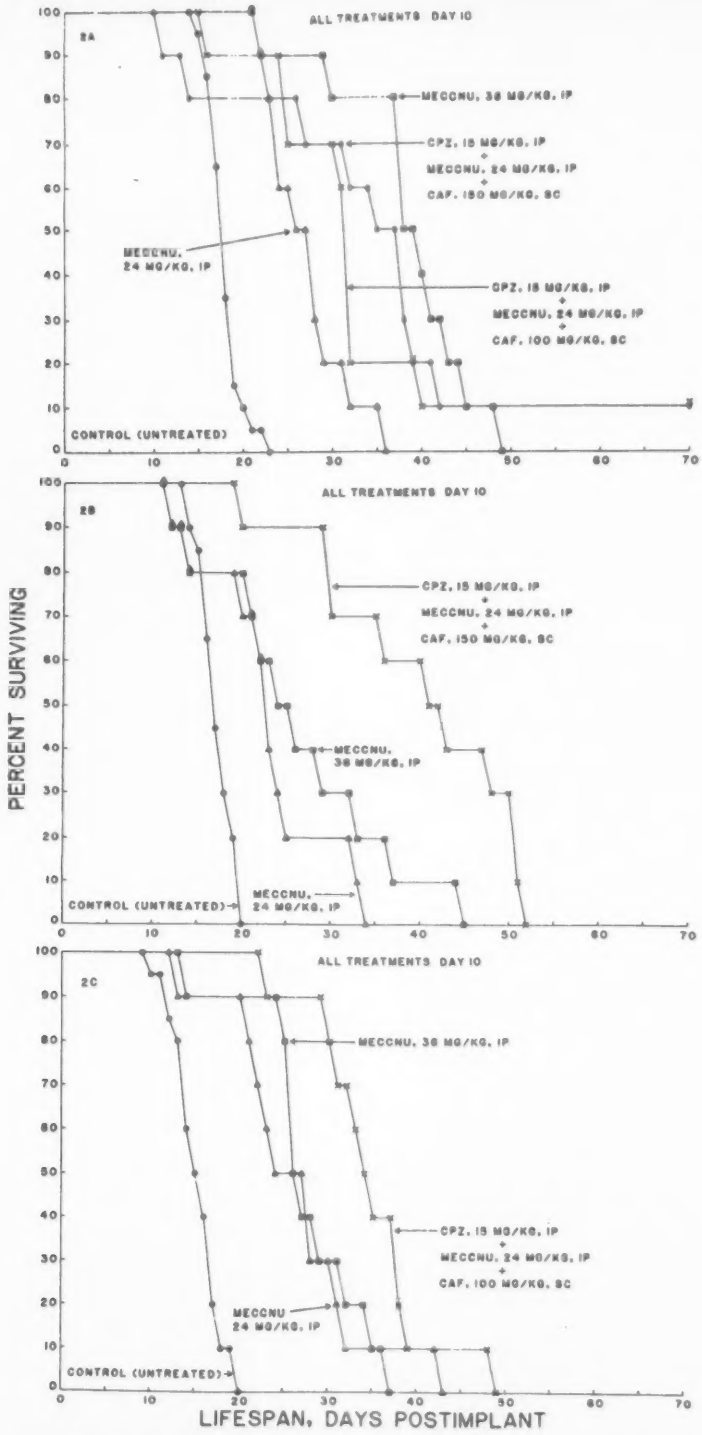

FIGURE 2.—Therapeutic potentiation of meCCNU by CPZ and CAF in mice implanted iv with 10^6 LL cells (10 mice/group except untreated controls, 20 mice).

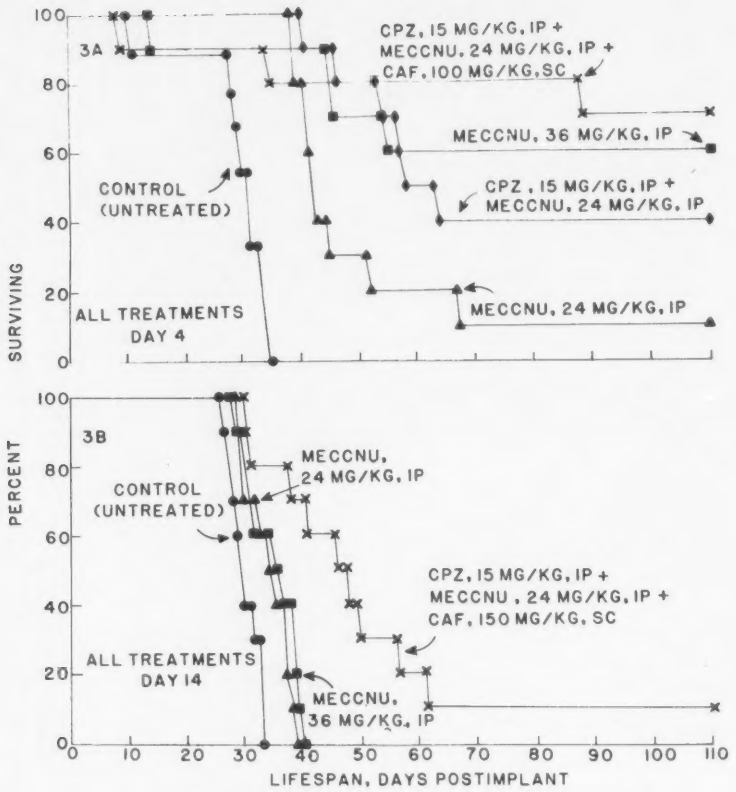

FIGURE 3.—Therapeutic potentiation of meCCNU by CPZ and CAF in mice implanted iv with 10^6 B16 cells (9 or 10 mice/group).

alone, 24 or 36 mg/kg, caused 67% and 70% ILSs, respectively. An MTD of 24 mg/kg of meCCNU plus 15 mg/kg of CPZ plus 100 mg/kg of CAF induced a 122% ILS.

B16 Melanoma

The triple-drug combination of meCCNU, CPZ, and CAF was evaluated in mice bearing either iv (fig 3) or sc (fig 4) implanted B16. In the first of two experiments in which B16 was implanted iv, treatment was administered on Day 4 only (fig 3A). A combination of 15 mg/kg of CPZ plus 150 mg/kg of CAF displayed no therapeutic activity. Single doses of meCCNU however, caused a 31% ILS and cured one of ten mice, or a 44% ILS with six of ten cures, depending upon whether 24 or 36 mg/kg, respectively, was used. Although the addition of 150 mg/kg of CAF had no effect on the therapeutic response achieved with meCCNU, the combination of 15 mg/kg of CPZ plus 24 mg/kg of meCCNU resulted in a response (70% ILS with four of ten mice cured) superior to that observed with 24 mg/kg of meCCNU alone. Better therapeutic responses however, were achieved using the three-drug combinations of 15 mg/kg of CPZ, 100 mg/kg of CAF, and either 24 mg/kg (fig 3A) or 30 mg/kg (not illustrated) of meCCNU. These regimens, which produced six to seven of ten cures and as much as a 63% ILS at MTD levels, were comparable in activity to the highest single dose of meCCNU evaluated: 36 mg/kg (also an MTD).

In the second experiment involving iv implanted B16, treatment was delayed until Day 14 after implantation (fig 3B). The disease had progressed to the point where meCCNU alone failed to cure any of the mice; in fact, 24 and 36 mg/kg of meCCNU caused only 8% and 15% ILSs, respectively. As in the first B16 experiment, a combination of 15 mg/kg of CPZ and 150 mg/kg of CAF failed to affect the MST of treated mice compared to untreated controls. MeCCNU at a dose of 24 mg/kg plus CAF at a

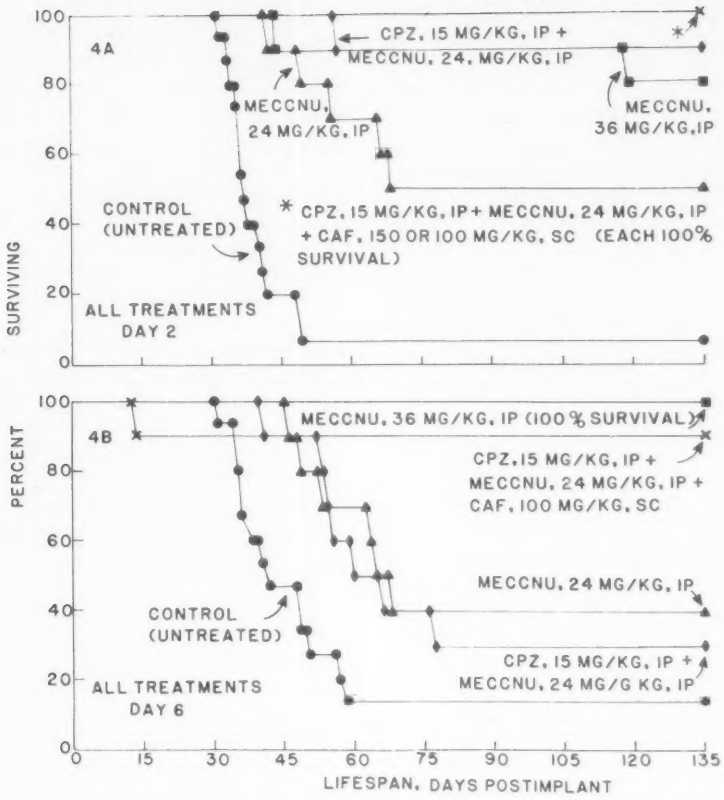

FIGURE 4.—Therapeutic potentiation of meCCNU by CPZ and CAF in mice implanted sc with 10^4 B16 cells (9 or 10 mice/group except untreated controls, 15 mice).

dose of 150 mg/kg caused only an 11% ILS and this same dose of meCCNU plus 15 mg/kg of CPZ caused a 17% ILS, neither response being different from those achieved using meCCNU alone. In comparison, the triple-drug combinations of 15 mg/kg of CPZ plus 24 mg/kg of meCCNU plus 150 mg/kg of CAF (fig 3B), or 15 mg/kg of CPZ plus 30 mg/kg of meCCNU plus 100 mg/kg of CAF (not illustrated), both yielded one of ten mice cured with ILSs of 49% and 45%, respectively. These responses were superior to those observed using meCCNU alone or any of the two-drug combinations evaluated.

Additional experiments involving meCCNU, CPZ, and CAF were performed using mice implanted sc with B16 (fig 4). In a pilot study (data not shown), we found that by themselves, neither CAF nor CPZ was therapeutically effective at MTD levels in mice bearing sc B16. In the first regular study, a minimal tumorigenic dose of 10^4 B16 cells caused 14 of 15 deaths (MST = 36.5 days) among untreated mice. Early treatment on Day 2 after implantation with 24 mg/kg of meCCNU resulted in five of ten mice cured and a 56% ILS among the dying mice; at a dose of 36 mg/kg of meCCNU, eight of ten mice were cured (fig 4A). As in the experiments in which iv implanted B16 was used, a combination of 15 mg/kg of CPZ plus 150 mg/kg of CAF failed to demonstrate any antitumor activity, and 24 mg/kg of meCCNU plus 150 mg/kg of CAF produced a therapeutic response (five of ten mice cured) comparable to that achieved with 24 mg/kg of meCCNU alone. The combination of 24 mg/kg of meCCNU plus 15 mg/kg of CPZ cured nine of ten mice (fig 4A) and this response was better than that achieved with 24 mg/kg of meCCNU alone but similar to that found with 36 mg/kg of meCCNU. When all three drugs were administered to tumor-bearing mice, cure rates of 100% were achieved (fig 4A).

In the final B16 experiment, mice bearing sc tumors were not treated until Day 6 after implan-

tation (fig 4B). Only 13 of 15 untreated tumor-bearing control mice died, with an MST of 41 days. Although 36 mg/kg of meCCNU cured ten of ten mice, 24 mg/kg of meCCNU cured only four of ten mice with a 47% ILS observed among those dying. The combination of 15 mg/kg of CPZ plus 150 mg/kg of CAF had no demonstrable antitumor effect, and 150 mg/kg of CAF plus 24 mg/kg of meCCNU caused a therapeutic response (two of ten mice cured and a 24% ILS) that was no better than that observed using 24 mg/kg of meCCNU alone. However, contrary to previous results, the combination of 15 mg/kg of CPZ and 24 mg/kg of meCCNU also induced a response (two of ten mice cured and a 45% ILS) that was no better than that achieved using only meCCNU. Finally, the triple-drug regimen of 15 mg/kg of CPZ plus 24 mg/kg of meCCNU and 100 mg/kg of CAF cured nine of ten mice (fig 4B).

DISCUSSION

The following conclusions can be drawn regarding the interaction between CAF and CPZ with either meCCNU or BCNU as evaluated in three murine tumor systems, L1210, LL, and B16: (*a*) neither CAF nor CPZ, singly or in combination with each other, demonstrated any antitumor activity; (*b*) meCCNU and BCNU were effective against the tumors evaluated and, except for BCNU against L1210, their activity was not increased by the addition of CAF; (*c*) combinations of these nitrosoureas plus CPZ resulted in an inconsistent therapeutic advantage against B16 (meCCNU), a consistent and definite therapeutic potentiation against L1210 (BCNU), and no therapeutic advantage against LL (meCCNU), when compared to the response obtained with the same dose of meCCNU or BCNU alone (as was used in the combination therapies); and (*d*) the combined use of all three drugs consistently yielded therapeutic responses greater than expected based upon the summation of the responses obtained with each individual drug at the same dose levels used in the combination therapies. In several instances, drug combinations achieved responses superior to those found using any dose of meCCNU or BCNU evaluated alone.

According to Venditti and Goldin (12), therapeutic synergism occurs when a drug combination produces a therapeutic response that is superior to the maximum response achieved with either drug alone. The therapeutic responses observed in mice bearing LL or B16 treated with MTD combinations of meCCNU, CPZ, and CAF did, at times, surpass that achieved using the MTD of meCCNU: 36 mg/kg. At other times, the use of 24 or 30 mg/kg of meCCNU with CAF and CPZ produced responses only comparable to the MTD of meCCNU. At doses approaching their MTDs, neither CPZ nor CAF was effective against L1210, yet BCNU plus either or both of these drugs always yielded responses superior to those obtained with any dose of BCNU alone. Regardless of whether the drug potentiations described resulted in therapeutic synergism (12) in all instances, with many of the combinations we clearly achieved optimal therapeutic responses with less nitrosourea. Considering the major debilitating effects of the nitrosoureas, gastrointestinal toxicity and myelosuppression (13), the cumulative damage caused by CAF and CPZ plus a reduced dose of nitrosourea may be preferable to that caused by an equitoxic dose of only meCCNU or BCNU, the former toxicity possibly being more manageable. In addition, prochlorperazine, a phenothiazine like CPZ, has been reported to inhibit the nephrotoxicity of meCCNU in mice (14).

In attempting to quantitate the magnitude of the therapeutic potentiation observed, specifically in the L1210 system, it is often useful to consider the MSTs of the dying mice after treatment as they relate to the MSTs of untreated leukemic controls receiving titrated numbers of leukemic cells, and the fraction of mice cured (10). For example, application of the Poisson distribution would lead one to calculate that when one of ten mice is cured, there remained an average of 2.3 L1210 cells/mouse following drug treatment of that group, and, should eight of ten mice be cured, 0.2 L1210 cell/mouse remained following drug treatment (15). Since these cure rates are among those observed following therapy with BCNU and BCNU plus CPZ plus CAF, respectively, one may conclude that the degree of therapeutic potentiation was about one order of magnitude. However, because of the consistently greater MSTs of dying mice found in the combination chemotherapy treatment groups, as compared to those receiving only BCNU, it is conceivable that > 1 log differences in leukemic cell kill were obtained than are assumed based upon the Poisson distribution.

According to the titration data obtained for each L1210 suspension used for experimentation, mice implanted with one L1210 cell died with MSTs of about 13–14 days. This is interpreted to mean that any group of mice with MSTs of 13–14 days after treatment (approximately 18 days after implantation in our L1210 experiments) probably had about one L1210 cell/mouse remaining following drug treatment (10). In the L1210 experiments performed, a few groups of mice receiving BCNU alone had MSTs of about 18 days after implantation, thereby suggesting that only one L1210 cell/mouse remained after treatment. Yet, a great many groups

of mice, in these same experiments, that received combination chemotherapy had MSTs of > 20 days (sometimes as much as 30 days) after implantation. We do not know why so many mice dying of L1210 leukemia following combination chemotherapy managed to live longer than expected if only one leukemia cell had survived drug treatment. We do know that almost 50% of mice cured with BCNU 3 days after implantation of 10^7 L1210 cells will survive a rechallenge of 10^3 L1210 cells (16). Is it possible that CAF and CPZ affected either the host or the L1210 population so as to allow the mouse's immune system to have a greater effect in perturbing the growth of, and/or destroying, residual L1210 cells following BCNU-induced cytoreduction?

Although CPZ has been reported to inhibit the growth of B16 in mice treated on a continuous schedule (5), we observed no evidence of an antitumor effect using a single MTD. Nevertheless, a therapeutic potentiation in mice bearing B16 was sometimes caused by the addition of CPZ to meCCNU therapy. CPZ is known to accumulate in higher concentrations in melanoma than in nonmelanized tissue (17) and has been described as inhibiting DNA synthesis in bone marrow granulocytes, resulting in a prolonged generation time and delay in cell division (18). It is not known whether CPZ inhibits DNA synthesis in B16 cells.

The role of CAF in the drug interactions is unresolved. CAF is considered by some to be an inhibitor of postreplication DNA repair secondary to damage caused by alkylating agents and irradiation (reviewed in ref 19). Yet, it is not apparent why the meCCNU-CAF two-drug combinations failed to exhibit therapeutic potentiation against B16 and LL if CAF-induced inhibition of repair of damage due to alkylation is a valid explanation of the triple-drug interaction.

With but minor disparity, our results confirm the observations reported by Cohen (1,2). We, too, were able to enhance the activity of BCNU against L1210 leukemia with the addition of CAF plus CPZ, but unlike Cohen we were also able to achieve therapeutic synergy with BCNU plus either CAF or CPZ. In view of the recent report by Driscoll et al (6), in which several psychotropic drugs were described as being active against both L1210 and P388 leukemias (whereas CPZ showed only inconsistent activity against L1210), perhaps better therapeutic potentiations could be achieved with those psychotropic drugs plus nitrosoureas as compared to CPZ plus nitrosoureas.

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Positive Phase II Trial of Dibromodulcitol in Patients With Metastatic Melanoma Refractory to DTIC and a Nitrosourea [1,2]

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SUMMARY

Twenty-five patients with measurable metastatic melanoma refractory to DTIC and a nitrosourea were treated with dibromodulcitol (DBD). DBD was administered orally at bedtime at a dose of 100 mg/m²/day until hematologic toxicity (a ≥ 50% decrease in the wbc or platelet count) was induced. Five patients experienced clinically useful objective remissions; responding lesions included both soft tissue metastases and visceral metastatic disease. It is concluded that DBD is useful in the treatment of patients with metastatic melanoma and thus joins DTIC and the nitrosoureas as single agents which are active against this malignancy.

[Cancer Treat Rep 62:2095–2099, 1978]

Dibromodulcitol (DBD) (fig 1), a halogenated hexitol, was chosen for clinical trial on the basis of significant antitumor activity in murine ascitic L1210 leukemia and Walker 256 carcinosarcoma.[7] Although originally thought to act as a bifunctional alkylator, more recent data suggest that DBD is a specific inhibitor of de novo DNA synthesis (1). Clinical trials have revealed activity against adenocarcinoma of the breast (2,3) and soft tissue sarcomas (2–4). The present study details a phase II evaluation of DBD in patients with metastatic melanoma refractory to DTIC and a nitrosourea.

MATERIALS AND METHODS

DBD (supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md) was administered to 25 patients with advanced measurable metastatic melanoma of nonocular origin. Patients with a life expectancy of < 3 months were ineligible, and a minimum of 28 days must have elapsed since the administration of any prior chemotherapy. Laboratory criteria for entry in the study were: hematocrit > 25%, wbc count > 4000/mm³,

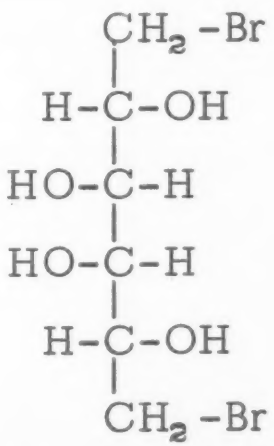

FIGURE 1.—Structural formula of DBD.

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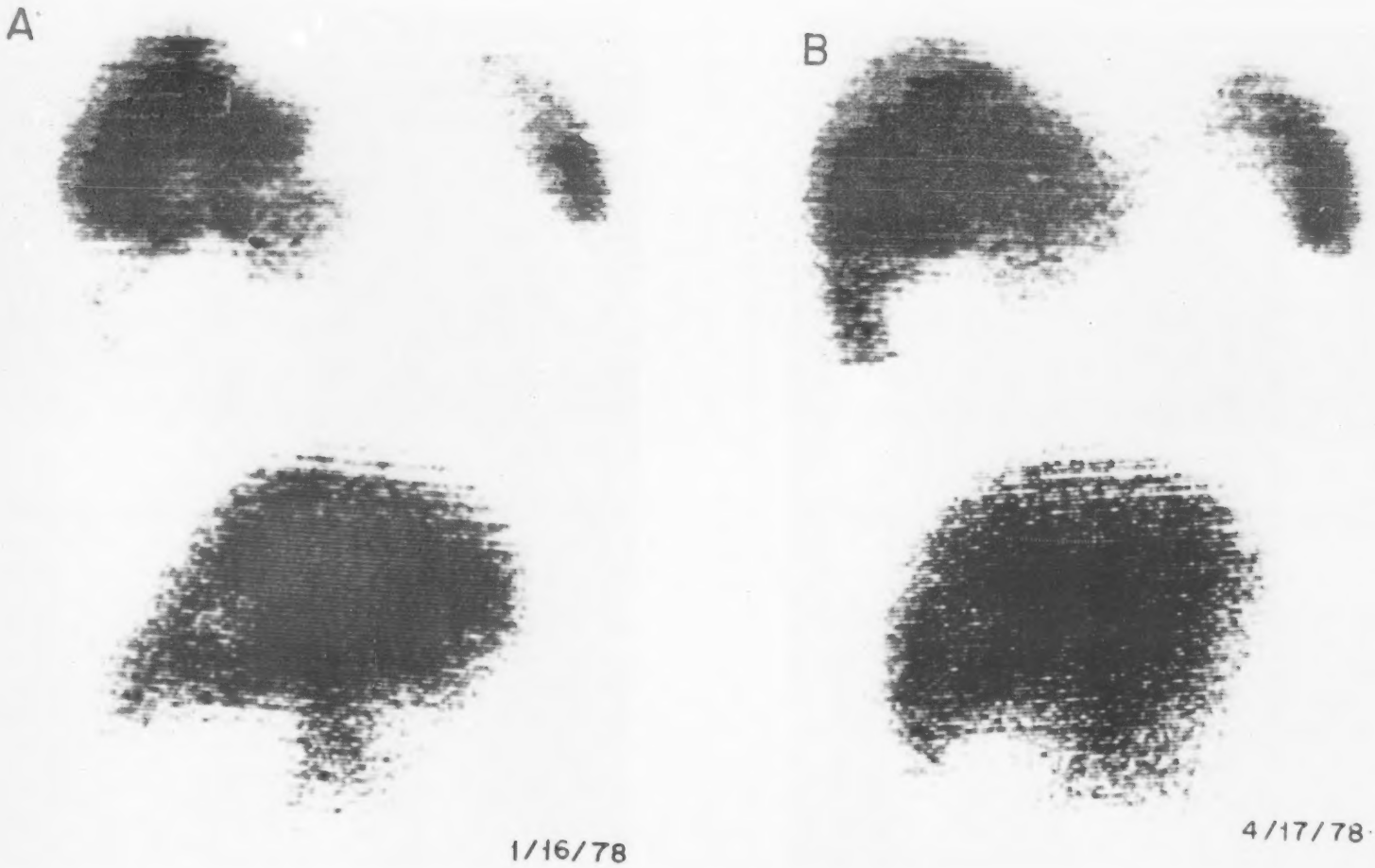

FIGURE 2.—^{99m}Technetium sulfur colloid liver-spleen scans of patient 4. (A) Pretreatment scan (anteroposterior [top], right lateral [bottom]) reveals multiple hepatic filling defects (serum LDH, 255 units [normal, 83–157]). (B) Posttreatment scan demonstrates significant improvement in isotopic uptake consistent with tumor regression (serum LDH, 135 units). During this time-frame, subcutaneous metastatic disease decreased in volume by > 90%.

TABLE 1.—Weekly DBD dose modifications

Wbc count (cells/mm³)		Platelet count (cells/mm³)	DBD dose (mg/m²)
≥ 4000	and	≥ 100,000	100
2000–3999	or	50,000–99,999	50
< 2000	or	< 50,000	0

platelet count > 100,000/mm³, BUN < 25 mg/100 ml, bilirubin < 1.5 mg/100 ml, and SGOT < 120 IU.

The patients included ten females and 15 males. The mean age for the females was 59 years (range, 42–72) and the mean age for the males was 54 years (range, 23–75). All patients had received prior systemic chemotherapy with DTIC and a nitrosourea. Four patients had also received VM-26 and four had received methotrexate. Two patients had failed to respond to melphalan and one patient had failed to respond to 5-azacytidine. Two males had previously responded to systemic chemotherapy with methyl-CCNU; one had a complete remission (CR) for 10 months and the other had a partial remission (PR) for 3 months.

DBD was administered orally at bedtime at a dose of 100 mg/m²/day (5) until hematologic toxicity (a ≥ 50% decrease in the wbc or platelet count) was induced. Cbcs and platelet counts were performed weekly with DBD dose modifications as shown in table 1. Patients experiencing objective tumor regression received daily maintenance therapy with DBD. BUN, total bilirubin, SGOT, alkaline phosphatase, and LDH determinations were performed at 35-day intervals.

Palpable dermal, subcutaneous, and lymph node metastases were measured at 5-week intervals; pulmonary parenchymal masses were evaluated with chest roentgenograms at 5-week intervals. Liver scans were performed at 10-week intervals. There were three response categories: CR, PR, and no response (NR). CR is defined as a regression of all measurable disease resulting in no evidence of melanoma for at least 1 month. PR is defined as a > 50% decrease in the sum of the products of the longest perpendicular diameters of all measurable disease (without progression of any lesion and without the appearance of new lesions) for at least 1 month. NR includes all other response categories.

TOXICITY

Of the 25 patients treated with DBD, only 20 achieved hematologic toxicity (table 2). Eleven patients developed a ≥ 50% decrease in the wbc count and 15 patients experienced a ≥ 50% decrease in the platelet count. The leukocyte and platelet nadirs (table 3) were noted on Day 49 (median) and on Day 35 (median) respectively. There was no evidence of sepsis as a result of leukopenia and no wbc count was < 1000/mm³. Three patients developed platelet counts of < 25,000/mm³; however, no patient had a hemorrhagic diathesis. Six patients developed anemia on the basis of bone marrow suppression requiring transfusion of rbcs. One patient complained of nausea; there were no episodes of emesis or diarrhea. Three patients reported somnolence secondary to systemic chemotherapy with DBD. There was no evidence of mucocutaneous, renal, hepatic, or pulmonary toxicity in any patient treated with DBD.

TABLE 2.—Toxicity of DBD in 25 melanoma patients

Toxic effect	No. of patients
Hematologic	20
Leukopenia (≥ 50% decrease)	11
Thrombocytopenia (≥ 50% decrease)	15
Anemia	6
Gastrointestinal	1
Nausea	1
Emesis	0
Diarrhea	0
Somnolence	3
Alopecia	0

RESPONSE

Five objective remissions were noted in the 25 patients treated with DBD: two CRs and three PRs. The clinical profiles of all five responders are shown in table 4. Median duration of response was 3 months (range, 2–7+). Median survival for nonresponders (measured from the start of systemic chemotherapy with DBD) was 3 months (range, 1–14+). For responders, the median survival has not yet been reached but will exceed 7 months. No statistically significant difference in survival is presently shown. Responding lesions included both soft tissue metastases and visceral metastatic disease (fig 2). No responder had experienced an objective remission to prior systemic chemotherapy.

DISCUSSION

In our extensive review of chemotherapy trials in patients with metastatic melanoma (6), we reported that three of 18 (16.7%) melanoma patients had responded to DBD (3,7). It was determined that DBD had a 90.2% probability of being at least 10% effective (ie, of having a true response rate [TRR] of ≥ 10%) considering this 16.7% objective response rate with a sample size of 18 patients (6). DBD was

TABLE 3.—Degree of hematologic toxicity in 25 melanoma patients treated with DBD

Wbc nadir (cells/mm³)	No. of patients	Platelet nadir (cells/mm³)	No. of patients
≤ 999	0	≤ 24,999	3
1000–1999	3	25,000–49,999	3
2000–2999	7	50,000–74,999	1
3000–3999	6	75,000–99,999	5
≥ 4000	9	≥ 100,000	13

TABLE 4.—Clinical data of 5 melanoma patients responding to DBD

Patient No.	Age/sex	Response	Duration of response (mos)	Metastatic disease	Prior chemotherapy
1	66/M	PR	2	Subcutaneous	DTIC, methyl-CCNU, VM-26, methotrexate, melphalan
2	53/F	CR	2	Lung	Methyl-CCNU, DTIC
3	42/F	PR	3	Lung	Methyl-CCNU, DTIC
4	64/F	PR	5+	Subcutaneous + liver	DTIC + CCNU
5	56/F	CR	7+	Skin + lung	Methyl-CCNU, DTIC

therefore categorized as *potentially useful* in the treatment of metastatic melanoma and worthy of further evaluation (6). On that basis, the present phase II clinical trial of DBD in patients with metastatic melanoma refractory to DTIC and a nitrosourea was initiated.

The toxicity data generated by the present study are consistent with those in previous reports (2–5). Gastrointestinal side effects were minimal and patient acceptance of this chemotherapeutic agent was excellent.

In evaluating the antitumor (phase II) activity of oral agents, we have been greatly concerned with the problems of irregular absorption from the gastrointestinal tract (8) and patient noncompliance (9). For this reason, patients should be treated to hematologic toxicity. That is, a determination of lack of activity (NR) should not be made until a ≥ 50% decrease in the wbc or platelet count is induced. Consequently, we feel it is justified to exclude from evaluation for response our five patients who failed to experience DBD-induced hematologic toxicity. On this basis, the present trial reveals five responders in 20 evaluable patients for an objective response rate of 25%. It was determined that DBD has a 98.9% probability of being at least 10% effective (ie, of having a TRR of ≥ 10%) considering a 25% response rate with a sample size of 20 patients (6). According to previously proposed criteria, DBD should be reclassified as active in the treatment of metastatic melanoma (6).

The ability of DBD to induce objective, clinically useful tumor regressions in patients previously exposed to DTIC and a nitrosourea is quite impressive. It has been the experience of others (10–12) that secondary treatment for metastatic melanoma is unproductive. In our separate phase II evaluations of adriamycin (13), ICRF–159 (14), 5-azacytidine (15), and VM–26 (16) (all utilized as tertiary treatments) we found no suggestion of antitumor activity. Tertiary treatment with DBD, however, has produced a clinically significant response rate in the present study. The responses of visceral metastatic deposits were also impressive.

Cerebral metastatic disease in melanoma patients is a major clinical problem. DBD is lipid-soluble and achieves a level in the cerebrospinal fluid that is approximately 50% that of the serum (17). In a study by the Children's Cancer Study Group (18), DBD and cyclophosphamide were compared for effectiveness in remission maintenance therapy for childhood acute lymphoblastic leukemia. Central nervous system leukemia developed in nine of 17 patients who received cyclophosphamide compared to one of 23 patients treated with DBD ($P = 0.001$). The potential of DBD to favorably affect central nervous system metastases may have a positive impact upon the survival of patients with advanced melanoma.

We are encouraged by the clinically useful responses shown in our phase II melanoma-oriented trial of DBD. On this basis, we have recently begun

a phase II trial of DBD in patients with metastatic melanoma previously unexposed to systemic chemotherapy.

CONCLUSION

A phase II trial of DBD in 25 patients with metastatic melanoma refractory to DTIC and a nitrosourea revealed a respectable objective response rate. Responses were clinically useful and patient acceptance of DBD was excellent. DBD is useful in the treatment of patients with metastatic melanoma and thus joins DTIC and the nitrosoureas (6) as single agents active against this malignancy. Further phase II trials of DBD both in previously untreated patients and in patients refractory to DTIC and nitrosoureas will better define TRRs for these classes of patients.

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Relationship Between Plasma Levels of Human Chorionic Gonadotropin and Tumor Growth During Chemotherapy for Human Choriocarcinoma Maintained in the Hamster Cheek Pouch [1,2,3,4]

Brief Reports and Preliminary Communications

Michael Knecht* and Roy Hertz [5]

Choriocarcinoma is a neoplastic disorder characterized by the production of the tumor marker, human chorionic gonadotropin (hCG). Plasma and urinary levels of hCG are generally considered to reflect the total burden of choriocarcinoma (1). However, the level of hCG during the initial phases of chemotherapy has rarely been correlated with changes in the actual amount of tumor tissue in the patient (1). In the present experiments, the BeWo strain (2) of human choriocarcinoma maintained by serial passage in hamsters was employed to attempt to relate plasma levels of hCG with tumor growth during chemotherapy. The effect of drug treatment on hCG levels and on tumor size was measured during the phase of rapid growth of the tumor. The therapeutic response to methotrexate (MTX) and to actinomycin D was observed.[6]

MATERIALS AND METHODS

The BeWo strain of human choriocarcinoma was inoculated into the cheek pouch of 4–6-week-old female Golden Syrian hamsters (Charles River Lakeview, Newfield, NJ), according to a previously described method (2). The lag phase of tumor growth averaged 8 days (range, 5–11 days). Tumors then began a 5–7-day period of rapid growth. The hamsters' immune response against human tissue was impaired by administering antihamster lymphocyte serum (ALS) prepared in rabbits (Microbiological Associates, Bethesda, Md). The immunocytotoxicity titer of ALS against hamster lymphocytes obtained from peripheral lymph nodes was 1:16,000 (manufacturer's data). Hamsters were given 0.1 ml of ALS three times per week sc during the lag phase of tumor growth and 0.2 ml/day sc during the phase of rapid tumor growth. Preliminary experiments indicated that this dose schedule optimized the extent of tumor growth that could be obtained as compared to animals not treated with ALS. At the end of lag phase, animals with tumors of approximately the same size (visual observation), consistency (firm, reddish-purplish color), and plasma hCG levels (1.0 μg of hCG/ml of plasma) were divided into saline controls and drug-treated groups.

Saline or drug treatment was given daily for 4 days (usually on Days 8–11 after inoculation). MTX was administered at a dose of 25 mg/kg ip and actinomycin D at a dose of 50 μg/kg ip. It has been previously shown that these doses inhibit tumor growth in this model (2). On Day 8, 10, 12, or 13 after tumor inoculation, each tumor was removed, freed of surrounding tissue and excess blood, and weighed. Animals were bled from the retro-orbital space immediately preceding tumor removal for measurement of plasma levels of hCG by radioimmunoassay (RIA).

The double antibody method was used for hCG RIA (3). All RIA tubes contained a final volume of 25 μl of hamster plasma in order to eliminate any nonspecific effect of plasma. Plasma from animals without tumors showed no detectable interference in the hCG RIA. hCG values were calculated by converting log versus logit standard curves to a linear regression curve of least squares and reading the unknowns from this "best-fit" curve. Dunnett's comparison of a control mean to each other group

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[6]Methotrexate was kindly supplied by Dr. E. W. Cantrall, Lederle Labs, Pearl River, NY, and actinomycin D was supplied by Sigma Chemical Co, St. Louis, Mo.

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TABLE 1.—Effect of drug treatment on tumor growth and plasma hCG concentration

Treatment	μg of hCG/ml of plasma ± SEM*	Tumor weight (mg ± SEM)*	hCG plasma concentration/g of tumor weight ± SEM†
Day 8, baseline values	0.78 (14)‡ ± 0.11	8.4 (14) ± 0.7	92.7 (14) ± 11.3
Saline control			
Day 10	7.73 (16) ± 0.64	55.9 (16) ± 5.0	157.0 (16) ± 19.0
Day 12	20.49 (17) ± 2.16	282.6 (17) ± 15.7	71.7 (17) ± 5.7
Day 13	44.19 (12) ± 7.34	410.0 (12) ± 20.5	109.4 (12) ± 14.5
MTX			
25 mg/kg/day on Days 8–11			
Day 10	8.23 (12) ± 2.82	18.6 (12)§ ± 2.7	376.9 (12)§ ± 89.5
Day 12	2.80 (17)§ ± 0.78	16.9 (17)§ ± 3.0	145.8 (17) ± 28.1
Actinomycin D			
50 μg/kg/day on Days 8–11			
Day 10	15.47 (11) ± 2.56	19.1 (11)§ ± 2.1	851.0 (11)§ ± 125.1
Day 12	2.97 (15)§ ± 0.79	27.3 (15)§ ± 5.9	109.3 (15) ± 15.8

*Statistics compare saline controls with treated hamsters on the same day after tumor implantation (eg, control Day 10 versus MTX and actinomycin D Day 10).

†Statistics compare Day 8 vs all other days; the ratio of the hCG plasma concentration/g of tumor weight was obtained from the measurement of the μg of hCG/ml of plasma divided by the tumor weight for each hamster.

‡Nos. in parentheses = No. of animals/group.

§$P < 0.01$.

mean (two-tailed) was used for statistical determination after an analysis of variance revealed that the data resulting from treatment with either MTX or actinomycin D differed significantly from control.

RESULTS AND DISCUSSION

Table 1 summarizes the effects of treatment with either MTX or actinomycin D on tumor growth and hCG levels. Day 8 values are representative for tumors which were divided into both control and treatment groups. Measurements on Day 10 show that although tumor weight was already significantly lowered in animals treated with either MTX or actinomycin D as compared to controls ($P < 0.01$), hCG plasma levels were not significantly different. The ratio of the plasma concentration of hCG per gram of tumor tissue rose significantly in drug-treated animals as compared to the Day 8 value ($P < 0.01$). Thus, hCG levels on Day 10 give the false impression of a much larger tumor burden in experimental animals if the plasma concentration of hCG is used as an indicator of tumor size.

By Day 12, tumor weight in control animals increased 34-fold as compared to Day 8, whereas the tumor weight of MTX- and actinomycin D-treated hamsters increased only two- and three-fold, respectively. Thus, tumor growth was inhibited by > 90% in MTX and actinomycin D animals on Day 12. hCG plasma levels decreased in drug-treated hamsters on Day 12 and the plasma concentration of hCG per gram of tumor was comparable to the Day 8 value; this contrasts with the apparent rise in plasma hCG per gram of tumor found on Day 10 in animals treated with either MTX or actinomycin D. Both tumor weight and hCG plasma levels were significantly higher in controls than in drug-treated animals on Day 12 ($P < 0.01$).

The hCG plasma concentration per gram of tumor tissue remained relatively constant from Day 8 to 13 after inoculation in controls. Both tumor weight and hCG plasma levels rose rapidly over this 5-day period. Viability of the tumor at Day 13 in control hamsters was still quite high as indicated by successful transplantation of the tumor to subsequent generations and an increase in tumor weight and hCG levels from Day 12. Although the contribution by the host of vascular endothelial tissue and connective tissue to the total area of the tumor must be appreciated, plasma levels of hCG over this time period do reflect the actual tumor size in hamsters not treated with chemotherapy.

The results described here after treatment of choriocarcinoma with chemotherapy agree with the data reported in tissue culture studies (4). The rise in hCG after chemotherapy has been postulated to be a result of an hCG release after cell lysis or a drug-induced increase in hCG synthesis (4). Other possible explanations, such as alterations in metabolism and renal excretion of hCG as a result of

chemotherapy, are less applicable since the same increase occurs in tissue culture and because plasma levels of hCG in the hamster are determined primarily by the rate of hormone production rather than by the rate of elimination (5). The decline in hCG levels from Day 10 to Day 12 after chemotherapy is presumably attributable to the termination of hCG synthesis after cell death.

The use of ALS in the hamster cheek pouch model has been reported to provide a more accurate assessment of drug effects on choriocarcinoma growth since the potential effect of the hamster's immune response against human tissue is suppressed (6). ALS also allows tumor viability to be maintained over several months (6). Thus, a means of studying relapses of malignant cells after chemotherapy is provided by this model. Information on hCG production by choriocarcinoma cells potentially more resistant to chemotherapeutic agents could thus be obtained. Further investigation is required to observe the ultimate effects of chemotherapy on the plasma level of hCG under these experimental conditions.

In conclusion, this study has shown that drug treatment can precipitate an increase in plasma hCG while inhibiting tumor growth. This effect must be considered in evaluating plasma levels of hCG as an indicator of tumor viability and growth during the early phases of chemotherapy for choriocarcinoma.

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Phase I Study of Neocarzinostatin in Children With Cancer [1,2]

Brief Reports and Preliminary Communications

Gaston Rivera,* Cathryn Howarth, Rhomes J. A. Aur, and Charles B. Pratt [3]

SUMMARY

Twenty-three children with advanced cancer refractory to conventional therapy received weekly iv doses of neocarzinostatin for 5 weeks. Doses were escalated from 500 to 6750 units/m²/week. Four types of toxic manifestations occurred: acute reactions consisting of shaking chills with or without fever and cyanosis (rigor), hypersensitivity, vomiting, and marrow depression. Evidence of oncolytic activity was limited to patients with acute leukemia in whom phase II trials at doses between 3000 and 4500 units/m² appear warranted.

[Cancer Treat Rep 62:2105–2107, 1978]

Neocarzinostatin is a new cytotoxic drug isolated from *Streptomyces carzinostaticus* by Ishida in 1964 (1). In in vitro studies, neocarzinostatin induces an inhibition of DNA synthesis (2–4), probably by its ability to interact with template DNA (5). In addition, it also induces DNA strand scissions (6,7), arrests cells in G_2 phase of the cell cycle (8,9), and causes mitotic abnormalities. Clinical trials with neocarzinostatin were first begun in Japan in 1965 (10–13). Based on these reports, the Investigational Drug Branch of the National Cancer Institute (Bethesda, Md) in 1976 initiated studies for the evaluation of neocarzinostatin. It was thought that the agent could be of potential usefulness considering (*a*) the activity shown against experimental animal tumors (14,15), most importantly against murine L1210 leukemia resistant to conventional agents such as methotrexate and 6-mercaptopurine, and (*b*) the reports of partial as well as complete remissions in Japanese patients with acute leukemia (10–13). The study we present here is a phase I trial performed exclusively in children.

PATIENTS AND METHODS

Twenty-three patients with a median age of 9 years (range, 1–22 years), whose diseases were progressive and resistant to conventional therapy, were admitted. Diagnoses included acute lymphocytic leukemia (11 patients), acute myelocytic leukemia (one), rhabdomyosarcoma (three), osteosarcoma (two), adrenal carcinoma (two), neuroblastoma (one), parotid carcinoma (one), Ewing's sarcoma (one), and lymphoepithelioma (one). On admission to the protocol, normal renal and liver functions and written informed consent of the patients' parents were required.

Neocarzinostatin was manufactured by Kakayu Antibiotic Research Co Ltd and provided by the National Cancer Institute. The drug was supplied in aqueous solution in 2-ml amber ampules. Each milliliter contained 1000 units of neocarzinostatin and 0.015 M acetic acid-sodium acetate buffer, pH 5.0 qs.

The starting dose in this study was 500 units/m². The schedule of administration was weekly × 5 and the route of administration was iv. The agent was given by slow injection over 10 minutes. Dose escalations were as follows: 500, 1000, 2000, 3000, 4500, and 6750 units/m²/week. No patient received more than one dose level. Leukopenia and thrombocytopenia were defined as < 3000 leukocytes/mm³ and $< 100{,}000$ platelets/mm³.

RESULTS

Toxicity (table 1)

Seventy-seven doses of neocarzinostatin at six different dose levels were administered to the 23 patients entered in the study. Toxicity was not encountered at the first two dose levels. Four types of toxicity were observed in patients who received

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TABLE 1.—Phase I trial of neocarzinostatin: toxicity data

Dose (units/m²/wk)	No. of patients	No. of doses	No. of patients with toxic reactions/No. evaluable			
			Acute reactions	Hypersensitivity	Gastrointestinal	Hematologic*
500	2	10	0/2	0/2	0/2	0/2
1000	3	8	0/3	0/3	0/3	0/1
2000	3	16	1/3	1/3	0/3	1/2
3000	5	16	2/5	0/5	2/5	1/3
4500	5	13	5/5	0/5	2/5	1/1
6750	5	14	4/5	0/5	3/5	3/3
Totals	23	77	12/23	1/23	7/23	6/12

*Only 12 patients were evaluable for hematologic toxicity.

higher doses. The first, acute toxic reactions, occurred in 12 of 23 patients; these children had acute reactions within 1 hour after receiving neocarzinostatin consisting of shaking chills with or without fever or cyanosis (rigor). The reactions were transitory and patients responded to iv administration of antihistaminics. On occasion, reactions occurred with the administration of the first dose of neocarzinostatin. These reactions, however, were most severe at the dose level of 6750 units/m². The second toxic effect was hypersensitivity (one of 23 patients); one child experienced a moderate hypersensitivity reaction with wheezing and facial edema after receiving a fifth dose at the 2000-unit/m² dose level. Treatment was discontinued and the patient recovered with antihistaminic therapy. With the third type, gastrointestinal toxicity (seven of 23 patients), seven patients had vomiting during or immediately following the administration of the drug. Diarrhea and mucosal ulcerations were not observed. The fourth type was hematologic toxicity (six of 12 patients); only 12 of 23 patients were evaluable for the development of drug-related hematopoietic toxicity. The 11 nonevaluable patients were children with leukemia who were pancytopenic on admission to the study. The number of evaluable children per dose level, as well as those who developed marrow suppression, is shown in table 1. Hematologic toxicity was first seen at the dose level of 2000 units/m². Six of the 12 patients developed hematologic toxicity with the onset occurring between Days 8 and 48. The median nadir of leukopenia was 1000 cells/mm³ on Day 31 and the median nadir of thrombocytopenia was 3000 platelets/mm³ on Day 26. Hematologic recovery was observed but could not be adequately assessed in all patients since most of them died with progressive disease. Other forms of toxicity such as stomatitis, alopecia, and hepatic or renal toxic effects were not observed. There were no drug-related deaths.

Response Data

Evidence of oncolytic activity such as a decrease in leukocyte counts and peripheral blast cells was limited to four of 12 patients with leukemia. Three of these children had acute lymphocytic leukemia and one had acute myelocytic leukemia. Although during the administration of neocarzinostatin the leukocyte counts remained reduced in patients who were responding, bone marrow remissions were not attained. Dose levels at which these responses were observed were ≥ 3000 units/m²/week.

DISCUSSION

As with most phase I clinical trials, the starting dose in this study was one third of the toxic-dose low in large animals and two or three patients received the lower dose levels. Once it was determined that there was no untoward toxicity, more patients received the higher dose levels. The minimum toxic dose, that is the first dose level at which toxicity occurs, was 2000 units/m²/week, and the maximum tolerated dose was 6750 units/m²/week. At the highest level, all patients evaluable for hematologic toxicity developed marrow depression and the character of the acute reactions was most severe. In fact, some patients, despite having evidence of an encouraging initial oncolytic response, refused further neocarzinostatin due to the severity of the acute toxic reactions. It was therefore determined that both myelosuppression and acute reactions represented dose-limiting toxic effects. Not all patients received five total doses of chemotherapy as originally planned (in some instances because of death due to progressive disease), and a few patients received more than five doses since some effectiveness was noted and no further forms of chemotherapy were available to them.

Recent information obtained in other initial studies with neocarzinostatin confirms the antileukemic effects of this agent. Griffin and co-workers reported responses in ten of 14 adult patients with acute nonlymphocytic leukemia, indicating that with doses ranging from 2000 to 8000 units/m²/day for up to 8–10 days the systemic toxicity elicited was minimal. Patients receiving the higher dose levels, however, were treated by constant infusions rather than by bolus injections (17). McKelvey and coworkers completed a phase I trial of neocarzinostatin in 60 patients with a variety of neoplasms. The doses of neocarzinostatin ranged from 500 to 6500 units/m²/day and the agent was given over 5 days as a constant infusion or by rapid intermittent administration (18). Only patients with leukemia responded to therapy. Better responses and a higher incidence of toxicity—in the form of myelosuppression and allergic reactions—were reported in the patients who received intermittent therapy. These data may indicate that acute toxicity can be reduced by the administration of neocarzinostatin by a constant infusion; however, the potential effectiveness of the drug may also be decreased.

From the results of the present study we conclude that phase II trials with neocarzinostatin appear warranted for patients with acute leukemia at dose levels from 3000 to 4500 units/m².

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Baker's Antifol in Children With Late-Stage Acute Lymphocytic Leukemia [1,2]

Brief Reports and Preliminary Communications

Ruprecht Nitschke, Teresa Vietti, Abdel Ragab, and Paul Dyment [3,4,*]

Baker's antifol (BAF) or triazinate is a triazine folate antagonist which inhibits dihydrofolate reductase (1). It interests the clinician for two reasons: (*a*) BAF penetrates the cell membrane unlike methotrexate (MTX), another dihydrofolate reductase inhibitor which requires an energy-coupled transport system (2). Therefore, it is potentially an active agent in tumors which lack the ability to transport MTX into the cells. (*b*) BAF crosses the brain barrier in dogs (3) and it may penetrate the blood-brain barrier in man (4). A phase II study in adults demonstrated that BAF is effective against lung adenocarcinoma, colorectal carcinoma, and renal cell carcinoma (5). The most frequent toxic effects were dermatitis, stomatitis, gastrointestinal symptoms, and mild myelosuppression (5). The Pediatric Division of the Southwest Oncology Group (SWOG) initiated the first phase II study with BAF for malignancies of childhood in 1976. The dose schedule used was the one recommended for adults (5). This paper describes the responses and toxic effects in children with late-stage acute lymphocytic leukemia (ALL) who were treated with BAF.

MATERIALS AND METHODS

Patients with ALL were eligible for study if they (*a*) were < 18 years old at the time of diagnosis and < 21 years of age at the time of entry in the study, (*b*) were in bone marrow relapse (M_3 marrow status), (*c*) were resistant to conventional therapy, (*d*) had not received chemotherapy for 1 week, (*e*) did not show any significant toxic effects from previous therapy, and (*f*) had an SGOT < 60 milliunits/ml and/or a bilirubin < 1.5 mg/100 ml and/or a creatinine < 1.5 mg/100 ml. Informed consent was obtained according to the guidelines of each institution.

BAF (250 mg/m^2) was infused over 60 minutes/day × 3 days in patients with normal liver and kidney function. If SGOT was between 40 and 60 milliunits/ml and/or if bilirubin and serum creatinine were between 1.0 and 1.5 and between 1.1 and 1.5 mg/100 ml respectively, BAF was reduced to 100 mg/m^2/day × 3 days. The courses were repeated every 3 weeks. Physical examination and cbcs were required weekly. A bone marrow aspiration and liver and kidney function tests were required before each new course of therapy. The criteria of the SWOG for bone marrow responses were used and are in accordance with the guidelines outlined in the SWOG procedures manual. To evaluate toxicity, one course and a survival of 7 days were required.

RESULTS

Eighteen patients were entered in this study. Their ages ranged from 3 to 19 years with a median of 7.1 years. The wbc count ranged from 450 to 52,000/μl with a median of 6300/μl. The number of previous relapses ranged from two to seven with a median of four. All patients were considered to be refractory to MTX. They previously received the drug as a component of maintenance therapy and relapsed while receiving it. Eight of the 18 patients had failed to respond to one or two phase II drugs for remission induction. Eleven patients had been maintained by ICRF-159, a phase II drug, before BAF administration. Only three patients had not received a phase II drug prior to BAF.

Nine of the 18 patients received one course and were in the study between 9 and 32 days with a median of 21 days. Six of these patients received 250 mg/m^2 and three received 100 mg/m^2. Of the nine patients, one patient died on Day 9, the parents of one patient refused further therapy, two patients

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[3]This study was performed by the Southwest Oncology Group. The following investigators participated in the study: *R. Nitschke* (University of Oklahoma Health Sciences Center, Oklahoma City); *T. Vietti* (Washington University School of Medicine, St. Louis, Mo); *A. Ragab* (Emory University School of Medicine, Atlanta, Ga); and *P. Dyment* (Cleveland Clinic Foundation, Ohio).

[4]We would like to express our appreciation to Marilyn Nelson for secretarial assistance in the preparation of this manuscript.

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showed no improvement, and five patients had progression of their disease.

Nine children received two courses. The children were in the study between 39 and 50 days with a median of 40 days. Five patients received 250 mg/m^2 for the first course. Because of toxicity the second course was reduced to 100 mg/m^2 in three of these patients. Four patients received 100 mg/m^2 for the first course. The second dose was increased to 250 mg/m^2 in only one patient. There were no responses. Seven patients had progressive disease; two patients showed no improvement.

TOXICITY

Three of the 18 patients had mild skin lesions and five had moderately severe skin lesions. These were manifested by generalized erythema, maculopapular rash, or erythema bullosum. Mucositis occurred in five patients; two patients had severe ulceration which prevented them from eating. The skin lesions and mucositis appeared 5 days after initiation of treatment and lasted from 2 to 8 days. During the days of drug administration, severe diarrhea occurred in two children. Mild vomiting was observed in two other children.

DISCUSSION

MTX, a potent drug against ALL, interferes with dihydrofolate reductase (as does BAF). The therapeutic failure of BAF may be due to cross-resistance to MTX, inadequate drug concentrations in the leukemic cells, or high BAF levels which are sustained too briefly in these cells. Data concerning cross-resistance between these two drugs in man are not available. All of our patients, as well as the few adults with ALL treated with BAF, were resistant to MTX (6). None of them responded to BAF. The BAF concentration in the leukemic cells may be the key factor in the effectiveness of this drug because it inhibits the dihydrofolate reductase at only one third of the rate that MTX does (6). The leukemic cells of two patients with ALL resistant to MTX were checked for the BAF levels (6). The BAF concentration in only one patient correlated well with the DNA inhibition. In the future, it may be of benefit to study in vitro BAF efficacy on leukemic cells which were not previously exposed to MTX, and compare the results with those of leukemic cells resistant to MTX (6).

CONCLUSION

BAF is ineffective in patients with late-stage ALL. The dose recommended for adults is tolerated by children. The main side effects are dermatitis and stomatitis.

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Melphalan and Methotrexate in Advanced Breast Cancer [1]

Brief Reports and Preliminary Communications

T. J. Priestman[2,3,*] and P. F. Salaman [2]

The combination of melphalan and methotrexate (MTX), given orally, has been selected for a multicenter study in the United Kingdom of adjuvant chemotherapy for women with early breast cancer (1). This report evaluates the activity of the combination in women with advanced breast cancer.

MATERIALS AND METHODS

All 20 patients had progressive and measurable locally recurrent or metastatic breast cancer. In addition, all patients had failed to respond to, or relapsed on, previous endocrine therapy. Seven women had received prior combination cytotoxic therapy, all having received alkylating agents. Six of the women had received cyclophosphamide and one had received chlorambucil. In two patients, the previous regimens had included MTX, and total doses of 50 and 300 mg respectively had been given prior to the present study.

All patients had local recurrence; in 13 patients this was the only site of disease. Three of the remaining patients had predominant bone metastases and one had bone and parenchymal lung deposits. In the three other women, lymphangitis, parenchymal lung secondaries, and liver involvement respectively were the dominant disease sites. All patients were ambulatory and received treatment on an outpatient basis.

Initially, each course consisted of melphalan at a dose of 5 mg twice daily and MTX at a dose of 5 mg daily given for 7 days, but subsequently the duration was reduced to 5 days. Courses were repeated at intervals of 4 weeks. If the wbc count fell to $< 3000/\mu l$ or if the platelet count fell to $< 100{,}000/\mu l$, the dose was reduced by 50%. If the wbc count fell to $< 2000/\mu l$ or if the platelet count fell to $< 70{,}000/\mu l$, the treatment was withheld until the counts recovered.

The criteria of response were those recommended by the UICC (2). Complete response indicated disappearance of all known disease (or radiologic evidence of calcification in the case of lytic bone disease). Partial response was a $> 50\%$ decrease in measurable lesions and objective improvement in evaluable but nonmeasurable lesions, with no new lesions and no progression of existing lesions. The criteria of the UICC do not specify a minimum duration in order for such regressions to be considered a response, but in the present study only those patients in whom remission was sustained for > 3 months were considered to have responded.

RESULTS

Twenty women were entered in the study between November 1975 and March 1976. The median age of the patients was 58 years (range, 38–73). The median interval from primary surgery to the first appearance of recurrence or metastasis was 13 months (range, 0–85). The overall response rate was 50% (table 1). The median duration of response was 7.25 months (range, 3–15). The median survival of responders was 18.5 months (range, 7.5–29+) and the median survival of nonresponders was 16.5 months (range, 8.5–26+).

The effect of prior therapy was as follows. Five of six patients who had previously responded to endocrine therapy responded on the present regimen, while only five of 14 who had no benefit from endocrine manipulation responded on the present regimen. Similarly, of those women who had previously responded to cytotoxic therapy, three of four gained a second remission, while one of three who had failed on more aggressive chemotherapy benefitted from melphalan and MTX.

Hematologic toxicity necessitating dose reduction was seen in five of seven patients receiving more than three courses of 7 days' duration. When the cycles were reduced to 5 days the incidence of marrow suppression was reduced; only three of 12 patients required dose reduction. Reducing the duration of courses had no demonstrable effect on efficacy. Subjective side effects comprised nausea (four patients), vomiting (one patient), oral soreness

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TABLE 1.—Response data

Site	No. of patients	No. of responses			
		Complete	Partial	Static disease	Progression
Overall					
Local disease only	13	—	6	1	6
Local + blood-borne spread	7	—	4	—	3
Individual sites					
Skin	15	—	9	1	5
Lymph nodes	8	—	3	1	4
Bone	4	—	1	—	3
Liver	1	—	—	—	1
Lung (parenchymal)	2	—	2	—	—
Lung (lymphangitis)	1	—	1	—	—

(one patient), and oral ulceration (one patient). Alopecia did not occur. In all but one patient, where nausea and vomiting were severe, subjective toxicity was mild and did not necessitate dose reduction. The average accumulated dose during the entire treatment period was 86% (range, 55%–100%) of the scheduled dose.

DISCUSSION

The overall response rate of 50% indicates that this regimen is active against adenocarcinoma of the breast, although no complete responses were seen and the durations of remission were short. Survival of responders was not significantly better than that of nonresponders, although the median survival times of the two groups (18.5 and 16.5 months respectively) are similar to those reported by Brambilla et al (3) with two more aggressive regimens (17.5 and 22.5 months). Most of the patients in the present series had predominant local disease and might therefore be expected to have a favorable prognosis. It must be emphasized, however, that these patients did have very advanced disease, all having received previous endocrine therapy and 35% also having been given prior cytotoxic treatment.

The combination of MTX and an alkylating agent in advanced breast cancer was initially described by Greenspan et al (4) using thioTEPA. In this instance, the thioTEPA was given im or iv and an objective response was seen in 15 of 40 patients (37%). Toxicity was significant and life-threatening in three patients. More recently, Hansen et al (5) included MTX and melphalan with CCNU and prednisone in an oral regimen for advanced breast cancer and reported a response rate of 56%. This was similar to the present study, and as in this study, they noted the lack of subjective toxicity of the regimen.

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Phase II Study of Mitomycin C and Vinblastine in Women With Advanced Breast Cancer Refractory to Standard Cytotoxic Therapy [1,2]

Brief Reports and Preliminary Communications

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SUMMARY

The combination of mitomycin C and vinblastine in the dose and schedule used in this study (6 and 5 mg/m², respectively, every 14 days) was of little clinical efficacy in the management of metastatic breast cancer already refractory to standard chemotherapy and hormonal therapeutic approaches.

[Cancer Treat Rep 62:2113–2115, 1978]

Mitomycin C (MMC) has been reported to be effective as a single agent in 0–44% (1–3) [mean, 33% (4)] of patients with metastatic breast carcinoma while the response rate for vinblastine (VBL) is cited at 0–45% [mean, 20% (5)]. Interpretation of these response rates is difficult because of variations from study to study in extent of disease, prior treatment, and dominant site of metastatic disease. The response rates to these agents in patients refractory to cyclophosphamide, adriamycin, methotrexate, 5-fluorouracil, and vincristine are not delineated. MMC has been shown to be non-cross-resistant to all other alkylating agents including cyclophosphamide in multiple in vitro systems (5) while VBL has shown some differences in mechanism of action from the other commonly used vinca alkaloid, vincristine (6). Vincristine and VBL also were additive when used in combination in a phase II trial of patients with lymphoma (7). Support for using MMC and VBL in combination is derived from in vivo L1210 mouse data showing that vincristine and VM-26 potentiate the effect of MMC (8). Clinically, the combination of MMC and VBL has been reported to produce objective responses in 33% of patients with advanced adenocarcinoma and large cell anaplastic carcinoma of the lung (9). With these data in mind and in an attempt to find effective combination therapy for patients with breast cancer refractory to standard cytotoxic therapy, a phase II trial of the combination of MMC and VBL in refractory breast cancer was initiated.

PATIENTS AND METHODS

Seventeen women (mean age, 52 years; median, 50 years) with histologically proven, advanced breast cancer refractory to standard chemotherapeutic regimens were entered in the trial. The mean duration of metastatic disease prior to MMC and VBL therapy was 19 months (range, 4–48 months). All patients had at least one measurable lesion and all had previously been treated with alkylating agents and other cytotoxic drugs (*see* table 1). The pretreatment absolute granulocyte count was $> 1500/mm^3$ and the platelet count was $> 100,000/mm^3$ in all patients. The Karnofsky performance status was $\geq 70\%$ in 16 of the 17 patients. No patient had received chemotherapy during the month preceding entry into the clinical trial.

The pretreatment evaluation included a complete history and physical examination, tumor measurements, performance status, cbc, platelet count, renal and liver function tests, carcinoembryonic antigen, chest roentgenogram, liver and bone scans, and roentgenograms of bone scan-positive areas. Cbc and platelet count were done weekly during therapy while tumor measurements and serum chemistries were repeated every 2 weeks. Bone roentgenograms were repeated every 6 weeks to follow lytic bone lesions. The definition of an objective response was standard and in conformity with the recently published guidelines of Hayward et al (10).

MMC and VBL were administered iv at a dose of 6 and 5 mg/m^2, respectively, every 14 days. This dose schedule has been found effective and tolerable in patients with large cell carcinoma and adenocar-

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TABLE 1.—Characteristics of 17 patients entered in the study

Characteristic	No. of patients (%)
Menopausal status (at time of first recurrence)	
Premenopausal	6 (35)
Postmenopausal	11 (65)
Dominant lesion	
Soft tissue	5 (29)
Visceral	9 (53)
Bone	3 (18)
No. of metastatic sites	
1 or 2	9 (53)
≥ 3	8 (47)
Prior hormonal treatment	10 (59)
Prior chemotherapy*	
C + F	17 (100)
C + A + F	16 (94)
C + A + F + M	15 (88)
C + A + F + M + V	8 (47)

*C = cyclophosphamide; F = 5-fluorouracil; A = adriamycin; M = methotrexate; V = vincristine.

cinoma of the lung (9). There was no concurrent antineoplastic treatment given.

RESULTS

Of the 17 patients entered in the study, 14 were evaluable for response. Of the three unevaluable patients, one died from drug toxicity after developing severe leukopenia (absolute granulocyte count of 400/mm^3) and fatal sepsis on the fifth day after the first treatment. The second patient was lost to followup after her first dose of chemotherapy. The third patient was the only patient entered in the trial within 1 month of termination of a hormonal manipulation. Although this patient had a partial response, it could not be determined whether this was due to the combination of MMC and VBL or to the withdrawal of tamoxifen which had been discontinued on the day of entry in the trial. Of the 14 evaluable patients, one patient had an objective response lasting for 16 weeks. In this patient, relapse occurred during a period of prolonged thrombocytopenia which precluded further drug administration. The average number of doses of combination therapy given to evaluable patients was 3.1, with seven patients receiving only two cycles of treatment before developing rapid disease progression. One patient had rapid progression of hepatic metastases after only one dose of MMC and VBL; he then received a cell-cycle specific combination chemotherapy regimen (11) and had a 6-month partial response.

The toxic effects of this combination of MMC and VBL were almost entirely hematologic and leukopenia was the primary toxic effect. There was one drug-related death as noted above. This patient had extensive metastatic disease involving the liver with a bilirubin level of 2.4 mg/100 ml which may have caused impaired excretion of VBL and subsequent toxicity. Including this patient, a total of nine of 16 (56%) patients considered evaluable for toxicity developed leukopenia. Six of these patients had grade 1 toxic reactions (< 1500 but > 750 absolute granulocytes/mm^3) and two patients had grade 2 toxic reactions (< 750 absolute granulocytes/mm^3 without sepsis). Four of 16 (25%) patients had platelet counts of 50,000–100,000/mm^3 (grade 1 toxicity) but no bleeding. Dose de-escalation was required in the responding patient but seven other patients would have required decreased doses if disease progression had not dictated a change in drug therapy after only two cycles. Two patients had prolonged granulocytopenia and thrombocytopenia lasting for 6 and 8 weeks, respectively. Nonhematologic toxicity was minimal. Only two patients developed moderate nausea and vomiting which was relieved with antiemetics. One patient developed a skin slough secondary to drug infiltration. There were no renal, hepatic, or neuromuscular toxic effects.

DISCUSSION

Patients with metastatic carcinoma of the breast who have failed to respond to primary and secondary cytotoxic therapy with combinations of cyclophosphamide, methotrexate, vincristine, adriamycin, and 5-fluorouracil have proven to be highly resistant to additional chemotherapy. In the present series, the combination of MMC and VBL was not effective in this heavily pretreated patient population. Our patients had received extensive prior treatment and had had metastatic disease for a mean of 19 months. Nearly one half of the patients had three or more metastatic areas while nine of 17 (53%) had visceral disease and three of 17 (18%) had bone disease as the principal site of metastatic disease. The only responding patient had soft tissue disease. A review of MMC reports indicates that some of the poorest response rates occurred in patients with extensive prior treatment (1). In studies reporting higher response rates for MMC, a majority of responding patients had primarily soft tissue metastatic disease. Wise et al (2) reported a 37% response rate (ten of 27 patients) for local, skin, or nodal disease while only two of 11 patients with liver disease and two of 14 patients with bone

disease as the primary site of metastatic involvement responded.

A review of the toxicity data shows that the primary effect was on the wbc count, reflecting that the dose of VBL which is primarily toxic to the granulocytic series was sufficient to cause toxicity. The limited amount of platelet toxicity, which in this combination would be expected to be caused by MMC, suggests that a higher initial dose may have been tolerated, but MMC also affects granulocytes and an increase in dose may have resulted in more severe granulocytopenia and infectious sequelae. It is expected that more severe toxicity would have been encountered if more than one half of the patients had not had disease progression after only two doses of MMC and VBL.

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Variable Oral Absorption of Hexamethylmelamine in Man [1,2]

Brief Reports and Preliminary Communications

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SUMMARY

Plasma levels of hexamethylmelamine were measured by a sensitive and specific gas chromatographic assay in 11 patients after they received oral doses ranging from 120 to 300 mg/m². Peak levels were reached in 0.5–3 hours and ranged from 0.2 to 20.8 μg/ml. The area under the curve ranged from 70.2 to 3606.6 μg/ml × minute. The half-life of the β-phase of elimination also showed considerable variability, ranging between 4.66 and 10.2 hours. These parameters were not related to the dose administered. No plasma accumulation of the drug was observed after daily treatment with hexamethylmelamine for 14–21 days.

[Cancer Treat Rep 62:2117–2119, 1978]

Hexamethylmelamine (HMM) is an antitumor agent active in different human malignancies (1,2); its mechanism of action is unknown, and despite the structural similarity with triethylenemelamine (TEM), it is not believed to act as an alkylating agent (3) or to have antifolic activity (4). The drug is poorly hydrosoluble and is therefore administered only orally.

Pharmacokinetic studies, mostly by Worzalla and Bryan et al (4,5), have identified the metabolites in plasma and urine of rats and man by gas chromatography-mass spectrometry. These same investigators have carried out studies by ultraviolet spectrophotometry or with HMM labeled in the ring with ^{14}C, but their method does not distinguish the drug from its metabolites.

The aim of the present study was to determine human plasma levels of HMM after oral doses of the drug, using a specific and sensitive gas-chromatographic assay.

MATERIALS AND METHODS

Measurements were made on 11 advanced ovarian carcinoma patients, ranging in age from 39 to 70 years, with normal liver and renal function. All patients had a performance status of 80–100. Five of these patients, who had never been treated before with antitumor agents, received a therapeutic protocol (OC/178) consisting of cycles of adriamycin (ADM) at a dose of 50 mg/m² iv on Day 1 followed by HMM at doses between 120 and 200 mg/m²/day orally and cyclophosphamide (CTX) at a dose of 100 mg/day orally for 14 days every 4 weeks. The other six patients, resistant to the previous therapy (ADM + CTX), became eligible for therapy with HMM only, at doses of 200–300 mg/m²/day orally for 21 consecutive days.

When the drug was given for the first time, 3-ml blood samples were taken from each patient (fasted for 5 hours before and after the treatment) at intervals up to 12 hours after the dose. HMM plasma levels were measured in the plasma of six of these patients 12 hours after the last dose of HMM, 14 or 21 days after the beginning of treatment.

Blood was transferred to heparinized tubes at 4°C and spun down at 1500 rpm; plasma was stored at –20°C. One milliliter of plasma was made alkaline with 50 μl of NaOH (0.6 N) and shaken in a horizontal position for 30 minutes with 10 ml of *n*-hexane (Carlo Erba). After 10 minutes of centrifugation at 2000 rpm, 9 ml of organic phase was transferred into another test tube and evaporated to dryness in a rotating evaporator. The residue was dissolved in 50–100 μl of methanol containing 20 μg/ml of TEM as external standard, and 1–2 μl of this solution was injected into a Fractovap 2300

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[5]We would like to thank Dr. Harry Wood, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md), for supplying the hexamethylmelamine and triethylenemelamine used in this study.

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TABLE 1.—Kinetic parameters of HMM after single or repeated treatment with HMM

Patient No.	Age (yrs)	Single treatment						Repeated treatment
		Dose (mg/m^2)	Peak level ($\mu g/ml$)	Time (hrs)	Level at 12 hrs ($\mu g/ml$)	AUC at 12 hrs ($\mu g/ml \times min$)	Half-life (hrs)	Level at 12 hrs after last dose ($\mu g/ml$)
1*	39	120	1.50	1	0.04	124.2	6.01	Not measured
2*	45	120	5.5	2	0.33	1265.4	4.66	0.03
3*	49	120	1.24	2	0.12	228.6	5.71	Not measured
4*	50	140	0.78	0.5	0.03	80.6	9.18	0.04
5	70	140	1.83	3	0.34	402.3	6.86	Not measured
6	51	140	0.63	1	0.17	178.2	10.2	0.2
7*	69	200	0.37	1	0.05	70.2	7.95	Not measured
8	51	200	20.8	1	1.20	3606.6	5.08	Not measured
9	52	300	0.71	1	0.06	112.2	6.53	0.05
10	57	300	0.20	2	0.12	113.2	8.96	0.08
11	44	300	1.62	1	0.04	135.1	4.9	0.03
Range	(39–70)	(120–300)	(0.2–20.8)	(0.5–3)	(0.03–1.2)	(70.2–2606.6)	(4.66–10.2)	(0.03–0.2)

* Patients who received ADM and CTX in combination.

(Carlo Erba) gas chromatograph with a 2-m glass column packed with 100–120-mesh Gas Chrom Q coated with 3% OV–17 and fitted with an NPSD detector. The calibration curve prepared with HMM standard is linear from 10 ng to 2 μg. (A detailed report of this method has been submitted for publication to *Analytical Biochemistry*.) ADM and CTX did not interfere with the analytic method for HMM assay. HMM metabolites were separated but their levels are not reported here. The experimental area under the curve (AUC) was calculated with the trapezoidal rule and the half-life by the Peeling method.

RESULTS

There were measurable levels of HMM in plasma in all patients up to 12 hours after administration and the drug was still detectable in patients tested at 24 hours (*see* fig 1). The peak plasma level of HMM was reached between 0.5 and 3 hours after treatment and ranged from 0.2 to 20.8 $\mu g/ml$ in the patients studied (table 1). There is no relation between the dose administered and the peak level, the AUC, or the level at 12 hours. The AUCs range from 70.2 to 3606.6 $\mu g/ml \times$ minute, about a 50-fold variation.

As can be seen in figure 1, the peak level was followed first by a rapid decline in plasma levels and then by a slower rate of disappearance. The half-life of the β-phase of elimination ranged from 4.66 to 10.2 hours; the mean value ± SE was 6.9 ± 0.57 hours. As shown in table 1, in patients tested 12 hours after the last dose of HMM there was no accumulation of the drug detectable in plasma in comparison to single-dose treatment.

DISCUSSION

Plasma levels of HMM in the ovarian carcinoma patients studied indicate that after oral administration, absorption of the drug varies widely. After doses ranging from 120 to 300 mg/m^2, no relation was found between dose administered and the peak plasma level or the AUC.

In studies in four patients, Bryan et al (5) found no unchanged HMM in human plasma, except in one patient where measurable levels were found only 2 hours after the high dose of 12 mg/kg of body weight. These investigators do not specify the sensitivity of their method based on column chromatography on anion and cation exchange resins followed by ultraviolet absorption spectrophotometry, but in the original paper in which the method is described (6), the calibration curve for calculation of recovery was plotted by adding to 2 ml of plasma large amounts of HMM ranging from 10 to 200 μg. If these are the conditions used, our patients too, at all times but one, would have given negative HMM plasma levels. In other studies in two patients, Worzalla et al (4), using HMM labeled on the ring with ^{14}C, found peak plasma radioactivity 1 hour after drug administration, and the plasma radioactivity half-life was 13 hours. The method employed by these investigators did not differentiate the unchanged drug from *N*-demethylated metabolites.

The present study cannot therefore be compared with other studies because it is the first time that a sensitive and specific method has been applied for the pharmacokinetic determination of HMM in man. We have established that the peak level of HMM in plasma ranges from 0.2 to 20.8 $\mu g/ml$ reached between 0.5 and 3 hours, that the AUC is from 70.2 to

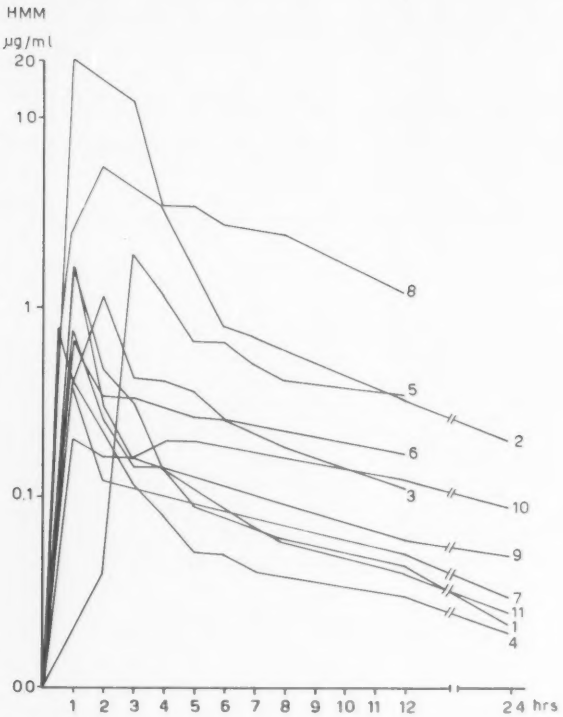

FIGURE 1.—Disappearance curves of HMM from plasma of 11 ovarian cancer patients given the drug orally at doses of 120 mg/m² (patients 1, 2, and 3), 140 mg/m² (patients 4, 5, and 6), 200 mg/m² (patients 7 and 8), and 300 mg/m² (patients 9, 10, and 11).

2306 μg/ml × minute, and that the half-life is from 4.66 to 10.2 hours.

Clinical studies have shown there is no difference in the response rate at two dose levels (4 and 8 mg/kg for 42 days) (7); this finding may be related to the high variability of plasma levels observed in this study. On the basis of our results, it is suggested that the measurement of HMM plasma levels might contribute to decreasing the variability in clinical response and perhaps eventually to improving the therapeutic efficacy.

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Testing of 2,3-Dihydro-1,3-6*H*-oxazine-dione (3-Oxauracil) in Mouse L1210 Leukemia and 37 Sarcoma [1]

Brief Reports and Preliminary Communications

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SUMMARY

The antimetabolite 2,3-dihydro-1,3-6*H*-oxazine-dione (3-oxauracil) has been investigated in an attempt to elucidate its effects on survival time of DBA/2 and B6D2F$_1$ mice with L1210 leukemia and of outbred albino mice with 37 sarcoma. A significant increase in survival time was observed in mice with 37 sarcoma and slight effects were observed in mice with L1210 leukemia when treated with 3 × 12.5 or 3 × 25 mg/kg of 3-oxauracil. No toxic side effects were observed when large therapeutic doses of the drug were given.

[Cancer Treat Rep 62:2121–2123, 1978]

2,3-Dihydro-1,3-6*H*-oxazine-dione (3-oxauracil), a pyrimidine antimetabolite, has been synthesized by Škoda et al (1) by a new method based on the oxidation of maleic monoamide with lead tetra-acetate in pyridine. The antitumor activity of 3-oxauracil in vivo was first tested by Ujházy et al in L1210 leukemia (2), due to its inhibitory effect on DNA and RNA synthesis of *Escherichia coli* in vitro (3,4). Another reason for Ujházy's study was that the drug expressed, in addition to antibacterial activity, some cytostatic effect on L51784, HeLa, Novikoff's hepatoma (5), and L1210 (6) cells in vitro.

This study tests further Ujházy's (2) observation that 3-oxauracil exerted a beneficial activity against L1210 cells in vitro and in vivo.

MATERIALS AND METHODS

Male DBA/2 and B6D2F$_1$ (C57BL/6 female × DBA/2 male) mice, purchased from Gödöllo Breeding Institute, Hungary, and male outbred albino mice, obtained from the Animal Production Unit, Oncological Research Institute, were used in these studies. The mice were 6–8 weeks old (20–25 g) at the time of tumor inoculation and were maintained under standard laboratory conditions. L1210 leukemia was maintained by continuous ip passage in syngeneic mice and was implanted in test mice according to the methods described by Geran et al (7). 37 sarcoma was maintained by continuous passage in BALB/c or outbred albino mice and was implanted in outbred albino mice according to the methods described by Sofina (8). In chemotherapy experiments, L1210 leukemia (10^5 cells) and 37 sarcoma (5×10^6 cells) were implanted ip.

3-Oxauracil was dissolved in sterile physiologic saline and the desired dose was administered ip in a volume of 1% of body weight. The treatment schedules were those used by Ujházy et al (2), and are reported in the Results section. There were eight mice per treated group and 18 mice per control group in each experiment. The experiments were carried out twice and gave similar results.

Antitumor activity was evaluated by determining the mean survival time of treated groups compared to control mice and was expressed as a percentage (% T/C). Statistical significance was assessed by the Student's t test.

RESULTS

The effect of 3-oxauracil on survival time of DBA/2 and B6D2F$_1$ mice with L1210 leukemia is shown in table 1. No prolongation of survival time was observed in L1210-bearing DBA/2 mice. A marginal activity of 3-oxauracil was observed in B6D2F$_1$ mice injected with either 12.5 mg/kg × 3 (T/C 126%) or 25 mg/kg × 3 (T/C 129.5%) in two separate experiments. No apparent toxicity was observed at any of the doses tested.

Table 2 shows the data on the effect of 3-oxauracil on survival time of outbred albino mice with ip 37 sarcoma in two separate experiments. The survival time (T/C) of the tumor-bearing mice increased in both experiments (from 161.4% to 218.8%) and again no toxicity was observed.

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TABLE 1.—Effect of 3-oxauracil on survival time of DBA/2 and B6D2F$_1$ mice with ip L1210 leukemia

Experiment No.	Mouse strain	Dose (mg/kg) Single	Dose (mg/kg) Total	Treatment (days)	Mean survival time (days)	T/C (%)	*P* value
1	DBA/2	—	—	—	9.6	—	—
		6.25	18.75	1–3	9.6	100.0	< 0.5
		12.50	37.50	1–3	10.6	110.4	< 0.05
		6.25	56.25	1–9	10.3	107.4	< 0.25
		12.50	112.50	1–9	10.3	107.4	< 0.5
2		—	—	—	7.6	—	—
		12.50	37.50	1–3	9.0	118.4	< 0.02
		25.00	75.00	1–3	9.1	119.7	< 0.001
		12.50	112.50	1–9	8.6	113.2	< 0.01
		25.00	225.00	1–9	9.2	121.0	< 0.01
1	B6D2F$_1$	—	—	—	9.8	—	—
		6.25	18.75	1–3	11.4	116.3	< 0.02
		12.50	37.50	1–3	12.4	126.5	< 0.01
		6.25	56.25	1–9	11.6	118.4	< 0.01
		12.50	112.50	1–9	11.0	111.2	< 0.1
2		—	—	—	8.0	—	—
		12.50	37.50	1–3	10.8	122.7	< 0.05
		25.00	75.00	1–3	11.4	129.5	< 0.01
		12.50	112.50	1–9	9.8	111.4	< 0.5
		25.00	225.00	1–9	10.3	117.0	< 0.1

TABLE 2.—Effect of 3-oxauracil on survival time of outbred albino mice with ip 37 sarcoma

Experiment No.	Dose (mg/kg) Single	Dose (mg/kg) Total	Treatment (days)	Mean survival time (days)	T/C (%)	*P* value
1	—	—	—	11.4	—	—
	12.50	37.50	1–3	18.4	161.4	< 0.02
	25.00	75.00	1–3	18.4	161.4	< 0.01
	12.50	112.50	1–9	16.0	140.4	< 0.01
	25.00	225.00	1–9	17.7	155.3	< 0.01
2	—	—	—	10.1	—	—
	12.50	37.50	1–3	16.7	165.3	< 0.05
	25.00	75.00	1–3	22.1	218.8	< 0.02
	12.50	112.50	1–9	17.1	169.3	< 0.25
	25.00	225.00	1–9	20.7	204.9	< 0.05

DISCUSSION

In the present study, both the 3- and 9-day treatments with 3-oxauracil did not significantly affect the survival time of L1210 leukemia-bearing DBA/2 mice. These results are in disagreement with the experiments of Ujházy et al (2), which were carried out in DBA/2 mice. The authors have observed a slight prolongation in the survival times of DBA/2 leukemia-bearing mice when the 3-day treatment schedule was applied at doses of 25 and 12.5 mg/kg, but the T/C increased > 200% after the 9-day treatment regimen. The reason(s) for this discrepancy is unknown. However, a host factor might be involved, since in the present study it was established that 3-oxauracil slightly prolonged the survival time of hemisyngeneic B6D2F$_1$ "leukemic mice" undergoing 3-day as well as 9-day treatment regimens (12.5 or 25 mg/kg). A more impressive result was achieved in the 37 sarcoma-bearing mice, where the maximum T/C value observed was 218.8%.

The conclusion to be made from the present study is that 3-oxauracil is an effective antimetabolite

with antitumor activity against both L1210 leukemia and 37 sarcoma. The lack of toxic side effects after large therapeutic doses are applied indicates that this drug could be beneficial in other experimental tumor model systems and eventually in human neoplasms.

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LETTER

A Case of Liver Toxicity Following *cis*-Dichlorodiammineplatinum(II) Treatment

cis-Dichlorodiammineplatinum(II) (DDP) is one of the most active cytotoxic agents developed in recent years (1). Nephrotoxicity, ototoxicity, and myelosuppression are well-known side effects of this drug. To our knowledge, liver toxicity has not as yet been reported. Pharmacokinetic studies in man have shown a high uptake of DDP in the liver (2,3), but the data do not ascertain whether or not biliary excretion takes place (4). We wish to report here on a patient who showed definite signs of liver toxicity after DDP administration.

A 47-year-old man had been subjected to a partial cystectomy for bladder carcinoma in mid-December 1976. Tumor tissue infiltrated the tunica muscularis (stage T_{3a}). Since DDP has been shown to be an active agent in the treatment of bladder carcinoma (5), we had instituted a protocol of adjuvant chemotherapy with this drug for "poor-risk" patients with bladder carcinoma. The above patient was entered into this study.

The protocol includes six cycles of DDP at a dose of 80 mg/m² iv every 4 weeks. The drug is given by

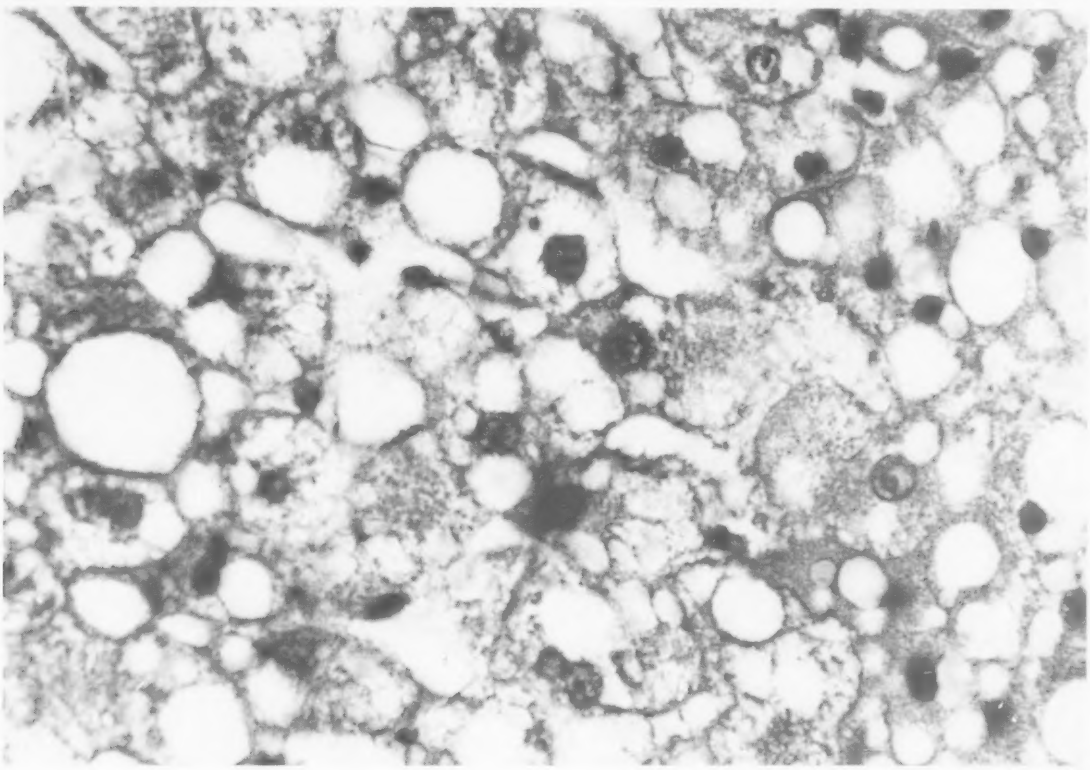

FIGURE 1.—Liver biopsy: the biopsy cylinder (12 mm in length; No. 26422–77) is composed of well-preserved liver parenchyma. The lobular structure of the tissue is normal; pseudoacini are lacking. There is a focal to diffuse, fatty change of hepatocytes (steatosis, moderate degree). Some parenchymal cells are swollen (intracellular edema), show a granular structure of the cytoplasm, and contain hemosiderin particles. Few hepatocytes are necrotic. A slight intralobular cholestasis can be observed. Signs of parenchyma damage are further documented by a rise of variation of nuclear size in hepatocytes and a diffuse increase in the number of Kupffer cells. Periportal tracts are edematous and show a light infiltration of lymphocytes and histiocytes. In summary, the changes are compatible with toxic liver damage.

iv push together with 40–80 mg of furosemide. Forced diuresis is carried out as previously described (4). Our patient received the first DDP dose on January 24, 1977. At that time, the values of the SGOT, the alkaline phosphatase, and the serum bilirubin concentration were normal. The patient had no family or personal history of liver disease. Four weeks later, the second DDP dose was omitted because the patient was slightly jaundiced. Laboratory studies showed the following values: total serum bilirubin, 2.22 mg/100 ml; SGOT, 58 units/100 ml (normal, to 18 units/100 ml); and alkaline phosphatase, 55 units/100 ml (normal, to 45 units/100 ml). The bilirubin was almost exclusively in a conjugated form. The search for hepatitis B surface antigen and antibodies repeatedly gave negative results. At the time of the third scheduled injection (March 21), all liver laboratory values, except the bilirubin with a value of 1.1 mg/100 ml, had returned to normal.

In the following months the patient received three further doses of DDP for a total of five doses. Each time we observed a similar course. One to 2 weeks after DDP administration, the serum bilirubin concentration rose to 3–6 mg/100 ml, the SGOT to three to five times its normal value, and the alkaline phosphatase up to twice the normal value. Four weeks later, in time for the next course, the liver enzyme values had reverted to normal. The bilirubin concentration always decreased considerably, but was within the normal range only before the fourth course; otherwise it remained slightly elevated (1.1–1.5 mg/100 ml) 4 weeks after the previous DDP dose. After the fifth course of DDP, the serum bilirubin concentration rose to 9.8 mg/100 ml and the SGOT to 144 units/ml. A liver biopsy was performed (*see* fig 1). It should be noted that the patient showed no nephrotoxicity during treatment with DDP. Myelosuppression was only moderate with a wbc count nadir of 2900/mm^3 following the third DDP dose and a platelet count nadir of 131,000/mm^3 after the fourth dose.

Since the clinical course and the histologic pattern were considered to be related to a DDP-elicited liver toxicity, therapy was stopped. We felt that this hepatotoxicity could not be explained by the other drugs given concurrently. The antiemetics varied from cycle to cycle. Furosemide was administered regularly with DDP. Although some cases of jaundice have been reported following the continual use of furosemide, we are not aware of any such hepatotoxicity associated with intermittent application of a few doses of this drug with long intervals between doses (6). Furthermore, Hill et al (3) reported transient elevations in the SGOT 5–14 days after DDP administration. This is suggestive of a potential DDP-induced liver toxicity. No pathologic data corroborating these enzymatic findings were presented.

At the last followup in December 1977 the patient was asymptomatic. There was no evidence of recurrence of bladder carcinoma. All liver function values were within normal ranges.

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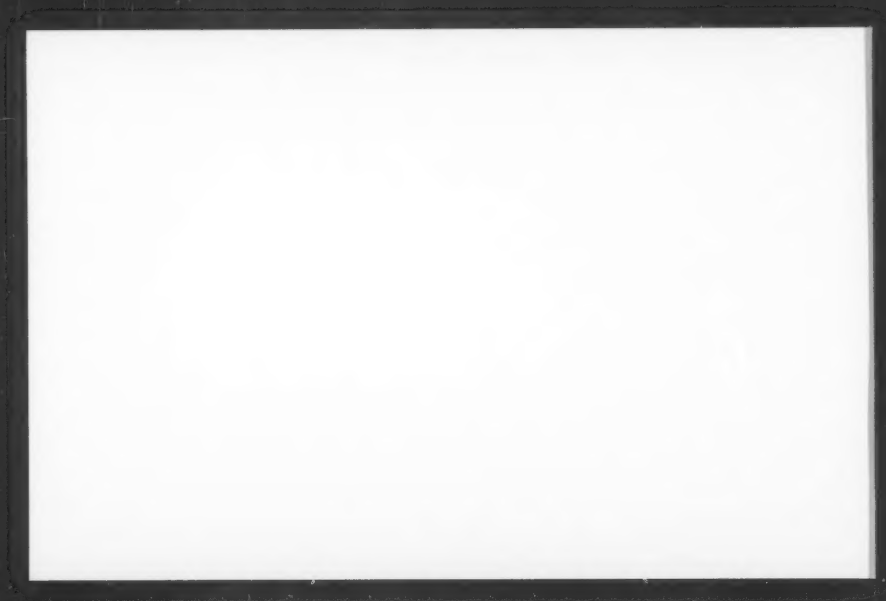

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Conference on Recent Advances in Oncology: Breast Cancer, Lung Cancer, and Non-Hodgkin's Lymphoma

Chapel Hill, North Carolina, April 27 and 28, 1979

The University of North Carolina is presenting a seminar in recent advances in Oncology. The directors of this meeting are Drs. James Newsome, Robert Capizzi, and Seth Rudnick. Subjects to be covered include recent advances in diagnosis, screening, and therapy for breast cancer, lung cancer, and non-Hodgkin's lymphoma. Guest speakers include Luther Brady, Bruce Chabner, Ronald Dorfman, Virgil Loeb, and James Wittliff. The course fulfills 9 credit hours of the Category I PRA-AMA award.

There will be a $90 registration fee. *For further information contact:* Office of Continuing Medical Education, University of North Carolina, School of Medicine, 319 MacNider 202H, Chapel Hill, NC 27514.

Seminars in Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be a seminar on "The Physician and Oral Cancer" on April 12, 1979, organized by the Department of Dentistry and Maxillofacial Prosthetics at Roswell Park Memorial Institute. The chairperson is Dr. Norman Schaaf.

There will be a $25.00 registration fee. *For further information contact:* Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

4th Annual "Symposium on Advances in Cancer Treatment Research"

Baltimore, Maryland, April 19–21, 1979

The Baltimore Cancer Research Center, National Cancer Institute, and the Program of Continuing Education of the University of Maryland School of Medicine are pleased to announce their 4th Annual "Symposium on Advances in Cancer Treatment Research," to be conducted in Baltimore, Maryland, April 19–21, 1979, at the Friendship International Hotel.

An experienced and internationally recognized faculty will participate. This Symposium is designed to update physicians and surgeons on some of the most recent developments in cancer treatment and current research including areas of clinical management, etiology, and cellular biology. Faculty members will be available throughout the Symposium to discuss any questions or interests of individual course registrants. A visit to the Baltimore Cancer Research Center can be scheduled for interested persons. The course is accredited for 16 hours of Category I credit for the PRA of the AMA by the Program of Continuing Education, University of Maryland School of Medicine.

For further information please contact: Jack Mason, PhD, Assistant Dean for Continuing Medical Education, 10 S Pine St, Rm 300E, Baltimore, Md 21201 (phone: 301-528-3956).

European Organization for Research on Treatment of Cancer Symposium on Advances in Cancer Chemotherapy

Institut Jules Bordet, Brussels, Belgium, October 18–20, 1979

The European Organization for Research on Treatment of Cancer (EORTC) announces a Symposium on Advances in Cancer Chemotherapy to be held October 18–20, 1979 (October 18–19: *Cis*-diamminedichloroplatinum, and October 19–20: free communications on cancer therapy) at the Institut Jules Bordet, Brussels, Belgium.

Free communications will be selected on the basis of submitted abstracts. Abstracts must be sent to Dr. M. Rozencweig, EORTC Data Center, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium, *before August 31, 1979.* Registration will be granted to the first 200 applicants. The registration fee is 3000 Belgian francs ($100 US). For further information, contact: Dr. M. Staquet, EORTC Data Center, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium (Phone: 538.57.90 - Telex: 22773).

2nd International Conference on the Adjuvant Therapy of Cancer

Tucson, Arizona, March 28–31, 1979

The 2nd International Conference on the Adjuvant Therapy of Cancer will be held in Tucson, Arizona, March 28–31, 1979, under the sponsorship of the Cancer Center Division of the University of Arizona. Drs. Sydney E. Salmon and Stephen Jones will serve as Conference Co-Chairmen. ***For further information, contact:*** Dorothy Baker, Conference Coordinator, Cancer Center Division, University of Arizona, Tucson, Ariz 85724 (telephone: 602/626–6044).

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